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Plecktrin Homology (PH) domains are commonly thought of as membrane-targeting modules involved in signaling pathways that bind phosphoinositides with high affinity and specificity. In a recent study of all PH domains in *S. cerevisiae*, only one bound PI(4,5)P2 with high affinity and specificity, while another six bound 3-phosphoinositides with moderate affinity and promiscuity; the remainder showed little or no affinity or specificity for phosphoinositides (Yu *et al*, 2004). All human PH domains were subdivided into 66 phylogenetic classes, and a "class representative" selected for *in vitro* phosphoinositide binding (21 completed) and *in vivo* localization studies (45 completed). The results are comparable to the yeast study, with only one confirmed high affinity and PI(4,5)P2-specific and several moderate affinity and promiscuous PH domains, while the remainder are low affinity and promiscuous. As in yeast, several low-to-moderate affinity and promiscuous PH domains showed plasma membrane or punctate localization. Two PH domains of this class possess comparable affinities for Golgi- and plama membrane-enriched phosphoinositides *in vitro*, although they both localize to the Golgi, not the plasma membrane *in vivo*. Additionally:

- A moderate affinity, PI(3,4)P2-specific PH domain was identified.
- An alkylphospholipid drug was found to selectively target a PH domain *in vitro*.

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Introduction

One of the primary motivations of my studies is to understand how PH domain recognition of phospholipids and of other proteins contributes to signaling by the wide array of molecules that contain PH domains – the 11th most common domain in the human genome, and one that is present in many proteins implicated in breast cancer (Cesareni *et al*, 2004). I wish to understand the nature of these PH domain interactions, and their regulation, in sufficient detail to suggest approaches for inhibiting the interactions pharmacologically. To pursue this aim, I am studying several PH domain/target pairs and I am employing biochemical, biophysical, and structural biological approaches to characterizing the interactions in detail. I will identify the binding site for the PH domain on novel targets using a combination of mutagenesis and *in vitro* binding studies. I will investigate the signaling capacity of the PH domain-containing protein *in vivo* when its PH domain can no longer interact with the newly identified target. I will also investigate the nature of the interaction quantitatively, and hope to co-crystallize the PH domain and its novel target in order to understand the structural basis for the recognition – and to allow structure-based drug design. By determining the structural basis of these interactions, and demonstrating a 'signaling relevance' to the interaction in breast cancer, I anticipate that these studies will succeed in identifying novel, highly-specific and well understood targets for disrupting intermolecular interactions. Since it appears that PH domains display properties of small molecule-binding modules and protein-protein interaction modules, I believe that they will offer a unique opportunity for disrupting critical protein-protein recognition events with small molecule agents that have many, many advantages as therapeutic agents.

Following the format of my proposed Statement of Work, my progress towards this aim is summarized as follows:

Task 1 Investigate affinity and specificity of phosphoinositide binding to isolated human PH domains (months 9-10)

For the initial part of this analysis, I have generated or collected GST-PH domain fusion constructs for 21 of the 66 representative human PH domains proposed for study, and I have analyzed their phosphoinositide binding specificity with a dot blot/lipid overlay assay, as previously described (Kavran *et al*, 1998; Yu *et al*, 2004). As expected from the concurrent analysis of yeast PH domains, the majority of the human PH domains selected bind phosphoinositides promiscuously and with only low affinity. The outcomes of these analyses for yeast (Yu *et al.*, 2004) and human are compared in **Tables 1 & 2**. Five of the human PH domains tested possessed high affinity and specificity for phosphoinositides (either for $PI(4,5)P_2$, $PI(3,4,5)P_3$, or $PI(3,4)P_2$/ $PI(3,4,5)P_3$). Only one yeast PH domain falls into this category. The two human PH domains of OSBP1 and FAPP1 form a separate group of domains that are promiscuous in their phosphoinositide binding, and have moderate affinity for these phospholipids when assessed using surface plasmon resonance studies.. The corresponding promiscuous, moderate affinity PH domains in yeast, Osh1p and Osh2p, are homologs of human OSBP1 (Lehto *et al*, 2001; Yu *et al*, 2004).

One outcome of these studies was the identification of a human PH domain with unusual phosphoinositide-binding properties. As shown in **Figure 1**, the PH domain of SH3-Binding

Protein-2 (SH3BP-2 or 3BP-2) is highly specific for $PI(3,4)P_2$, but unlike all other PI 3-kinase product binding PH domains, it binds this phosphoinositide with only moderate affinity. SH3BP-2 is an adaptor protein with a versatile role in a variety of cell types. It is in the same phylogenetic PH domain class as that of DAPP1 (one of the five $PI(3,4)P_2$/ $PI(3,4,5)P_3$-specific, high affinity binders), and is translocated to the plasma membrane upon PI3K activation (http://www.signaling-gateway.org/). These results are consistent with the protein's PI3K-dependent roles as a positive regulator of IL-2 gene induction in T cells (Deckert *et al*, 1998), NK cell-mediated cytotoxicity (Jevremovic *et al*, 2001), and FcåRI-induced degranulation and signal amplification in mast cells (Sada *et al*, 2002).

Task 2 Determine the subcellular localization of the PH domains (months 1-16).

Since the commonly accepted function of PH domains is to direct their 'host' protein to specific membranes in the cell, one might infer that PH domains with high, but not low, affinities for phosphoinositides should be membrane-localized *in vivo*. We strongly suspected this would not be true universally for all human PH domains, as several low affinity PH domains showed a surprising degree of localization to various cellular membranes in our yeast genomewide study (Yu *et al*, 2004). In my original proposal, I proposed an *in vivo* fluorescence localization study of human GFP-PH domain fusion constructs. Over the past year, the Alliance for Cell Signaling (AfCS) laboratory at Stanford has made significant progress in such a study of mouse PH domain localization in two separate mouse cell lines (72 in WEHI-231 cells and 148 in RAW 264.7 cells) (http://www.signaling-gateway.org/). This study has provided *in vivo* localization data for 45 of the 66 human PH domains classes listed in our original proposal (including 18 of the 21 PH domains that I have tested for phosphoinositide binding)(**Tables 2 & 3**). The overall distribution of cytosolic versus membrane- or puncta-localized PH domains is comparable to that of our yeast PH domain study. As with the yeast study, several human PH domains with low affinities for phosphoinositides *in vitro* demonstrated plasma membrane or punctate localization *in vivo*.

Since this work by the AfCS has accelerated my project considerably, I have been able to initiate studies focused on the poorly understood group of PH domains that localize to cellular membranes in spite of only moderate affinity for phosphoinositides and promiscuous lipid binding. To this end, I have chosen to characterize in detail the PH domains from the oxysterol-binding protein-1 (OSBP1) and Four-Phosphoinositide Adaptor Protein-1 (FAPP1), proteins that are involved in coordinating budding and fission events at the Golgi for the generation of cargo transporters targeted for fusion with the plasma membrane (Itoh & De Camilli, 2004; Roth 2004). My studies have yielded three interesting results thus far:

First, *in vivo* fluorescence of the GFP-PH domain fusions of OSBP1 and FAPP1 was punctate in nature (**Figure 2**), consistent with Golgi membrane localization, as found in previous studies (Levine & Munro, 2002; Godi *et al*, 2004; Balla *et al*, 2005).

Second, I found that the PH domains of OSBP1 and FAPP1 display *in vitro* binding affinities that are comparable for PtdIns(4)P (K_d 3.5 and 21 ìM, respectively) and $PtdIns(4,5)P_2$ (K_d 3.3 and 17 ìM, respectively), as measured by SPR (**Figure 3**). These data appear to be at odds with the *in vivo* localization data, which demonstrate clear PH domain targeting to the PtdIns(4)P-

enriched Golgi and not to the $PI(4,5)P_2$-enriched plasma membrane. How are these PH domains targeted to the correct site in the absence of major differences in their affinity and selectivity for phosphoinositides? This question is *apropos* for all PH domains that bind phosphoinositides with low affinity affinity and promiscuity that are nevertheless targeted to membranes and/or puncta.

Third, in a preliminary SPR competition assay, I showed that the PH domains of OSBP1 and FAPP1 could not be displaced from a $PI(4,5)P_2$-coated chip by the phosphoinositide's inositol headgroup, $Ins(1,4,5)P_3$. By contrast, well-known phosphoinositide-specific PH domains (such as the phospholipase C-δ_1 PH domain) are efficiently displaced by lipid headgroup. This finding argues that inositol headgroup contacts with the PH domain alone cannot account for Golgi membrane localization, and additional contacts are necessary - possibly with the phosphoinositide's acyl chains, and membrane insertion may occur. This type of nonspecific acyl chain binding has been demonstrated for the phosphoinositide-binding proteins gelsolin (Janmey *et al*, 1987), AP-2 (Gaidarov *et al*, 1996), AP-3 (Hao *et al*, 1996), centaurin-á (Hammonds-Odie *et al*, 1996), CAPS (Loyet *et al*, 1998) and dynamin (Burger *et al*, 2000), to name a few. These results contrast with those of prior studies on high affinity and phosphoinositide-specific PH domains, which are effectively displaced by inositol head group (Lemmon *et al*, 1995; Takeuchi *et al*, 2000), suggesting that the phosphoinositide headgroup accounts for the bulk of the specific interaction with the PH domain.

Task 3 Screen for putative interacting proteins of PH domains

Since phosphoinositide binding alone cannot account for the specific subcellular localization of several PH domains – particularly in the case of the Golgi-targeted OSBP PH domain, it has been suggested that other targets, particularly protein targets, may define their localization. For example, Levine and Munro observed that Golgi targeting of the OSBP1 PH domain requires both PI(4)P and a second determinant, which they suggested from genetic studies might be Arf1, a Golgi small GTPase (Levine & Munro, 2002). More recently, the PH domains of both OSBP1 and FAPP1 were found to specifically and directly interact with Arf1 *in vitro* and *in vivo* (Godi *et al*, 2004). I have recently obtained expression constructs for myristoylated Arf1 (**Figure 4**) from Paul Randazzo's lab, and I have established production and purification procedures (Randazzo & Fales, 2002). In the next project period I will quantify the association of the OSBP and FAPP1 PH domains with this small GTPase using SPR protein binding assays, analytical ultracentrifugation, and ultimately crystallographic studies that will allow a structural understandingof the putative complex and will allow mutations to me made in order to assess the functional consequences of disrupting this mode of membrane targeting.

PH domains as drug targets in cancer therapy

Our original view of PH domains suggested that phosphoinositide-binding PH domains themselves should make poor targets for pharmacological intervention, since most phosphoinositide-recognition events are essentially the same, and, moreover, drugs likely to target PH domains are very highly charged, which leads to delivery problems. Recently, I was presented with a unique opportunity to test the PH domain-binding properties of perifosine, a C_{18}-alkylphospholipid drug that has recently completed phase I trials (Van Ummersen *et al*, 2004; Crul *et al*, 2004) as an anti-cancer agent. Earlier immunoprecipitation studies suggested

that perifosine specifically inhibits Ser/Thr phosphorylation and kinase activation of Akt1/PKB *in vivo* and *in vitro* (Kondapaka *et al*, 2003). Myristoylated Akt1/PKB, which is targeted directly to the plasma membrane in a PH domain-independent manner, is unaffected by perifosine treatment. It was therefore hypothesized that perifosine might act by directly interfering with the phosphoinositide binding (or other membrane-targeting interaction) of the PKB PH domain. I have recently completed a series of SPR binding studies suggesting that perifosine specifically competes with phosphoinositides for binding to the PH domain of Akt1/PKB (EC_{50} 26 ìM), while it competes substantially less for binding to the PH domain of PLCä, and not at all for the PH domains of DAPP1 (**Figure 5**) and FAPP1 (data not shown). These studies indicate that perifosine may bind directly to the phosphoinositide-binding site of the PKB PH domain, and I am currently in the process of further analyses to determine both the strength of this interaction (using isothermal titration calorimetry) and its structural basis (using X-ray crystallography).

Key Research Accomplishments

- GST-PH domain fusion constructs (21 of the 66 representative PH domains) have been tested for their phosphoinositide affinity and specificity *in vitro* by dot blot and SPR(BIAcore) assays. The overall distribution of high, moderate, and low affinity PH domains in the human proteome appears to be comparable with that of yeast.
- GFP-PH domain fusion constructs of several moderate affinity and promiscuous PH domains were tested for *in* vivo localization in MDA-MB-468 and NIH 3T3 cells, demonstrating punctate localization.
- A unique PH domain (SH3BP-2) with moderate affinity, yet high specificity for $PI(3,4)P_2$ has been characterized.
- An alkylphospholipid drug has been identified as highly specific for the PH domain of Akt1/PKBα to the exclusion of several other related PH domains.

Reportable Outcomes

- Publications include contributing authorship in peer-reviewed journal and book chapter review of PH domains (Yu *et al*, 2004; Cesarini *et al* (ed.), 2004).
- Abstract and poster for upcoming Era of Hope Meeting.
- GST- and GFP-PH domain fusion constructs for many of the representative PH domains that had been originally proposed have been collected or cloned.
- Complete phylogenetic tree (dendrogram) of the entire human and yeast PH domain proteome.
- Complete phylogenetic tree (dendrogram) of other protein-binding domains of the human proteome including PTB, EVH/WH1, and RanBD

Conclusion

Through a combination of *in vitro* phosphoinositide binding and *in vivo* localization studies, a picture of the human PH domain proteome has emerged that is consistent with our previous yeast genomewide study (Yu *et al*, 2004). The vast majority of PH domains, as in yeast, are low affinity and promiscuous for phosphoinositides. I have observed only a handful of high affinity and phosphoinositide-specific human PH domains, while yeast have only one. An intermediate, relatively small group of PH domains possess moderate affinity and promiscuity for

phosphoinositides, and are related to the the yeast PH domains of Osh proteins, including OSBP and FAPP family members (Lehto *et al*, 2001). Most are targeted to the Golgi *in vivo*, and likely interact with both phospholipids and protein targets (Levine & Munro, 2002; Godi *et al*, 2004; unpublished data). Interestingly, at least one member of each family (FAPP2 and OSBP2) have been associated with brest cancer development or progression (Fournier *et al*, 1999; Scanlan *et al*, 2001). This family will be further characterized both structurally and functionally by the next reporting period.

During the course of my *in vitro* phosphoinositide binding study, I have identified SH3BP-2 PH as a unique $PI(3,4)P_2$-specific, moderate affinity PH domain. I may identify other PH domains with unusual phosphoinositide binding properties as the study continues. Additionally, I have demonstrated that the anticancer drug perifosine, recently having completed phase I clinical trials, competes with phosphoinositides for binding in a specific manner to PKB PH. Understanding the structural basis for this PH domain-specific interaction will allow me to elucidate the determinants involved, contributing to the quest for an effective anticancer therapy.

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Appendix

Table 1 Yeast PH Domains

Num1p		
Cla4p	Boi1p	
Skm1p	**Boi2p**	
Osh1p	***Osh2p***	
Ask10p	Syt1p	Caf120p
Bem2p	Ugt51p	Gcs1p
Cdc24p	Ybl060p	Opy1Ap
Opy1Bp	Yhr131p	Tus1p
Osh3p	**Yil105cp**	Yhr155wp
Sip3p	Ylr187wBp	Ynl144p
Spo14p	**Ynl047cp**	Ypr091cp
Spo71Bp	Ypr115wp	Spo71Ap
Stt4p	Bem3p	Ylr187wAp

Table 2 Human PH Domains

PLCä$_1$		
Tiam1-Ct	DAPP1 / PHISH	
Cytohesin 2 / ARNO	*TAPP1*	
*FAPP1**	*OSBP1**	
Vav1	AP20 / LL5â	PLD1
Dbl	FGD1-Nt	**Dok1**
Sos1	*FGD1-Ct*	Pleckstrin-Nt
IRS1	**DAGKã**	Dynamin 1
âARK1	KIAA0053, RhoGAP25	Myosin X-Nt

Table 3 Mouse PH Domains (Localization)

PM localization	Cytosolic/Nuclear	Nuclear	Puncta
Lbc / Ht31	Tiam1-Ct	JBP / TNFidp	Kif1a
Gab2	Grb7		TAPP2-Nt
Phafin 1	Net1		
Spectrin â2	PKCμ / PKD		
ORP3	Oligophrenin 1		
CNK2	Centaurin δ_2 / ARAP1		
	GNRP-Nt		
	GNRP-Ct		
	Trad / Duet		
	NGEF		
	Rasal (RasGAP)		
	CAPS		
	Tec		
	SH2â		
	TAPP2-Ct		

Table 1 Yeast PH domain affinity *in vitro* and localization *in vivo*

Color: High affinity and PI(4,5)P_2-specific in red, High affinity and PI(3,4)P_2/PI(3,4,5)P_3-specific in green, Moderate affinity and promiscuous in purple, Low affinity and promiscuous in blue. Font: Cytosolic and nuclear localization is regular, Plasma membrane localization is **bold**, Punctate localization is *italicized* (see Yu *et al*, 2004 for details).

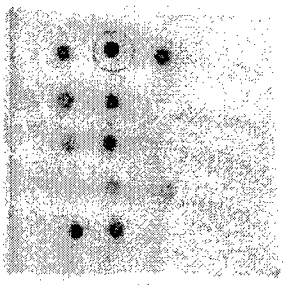
Table 2 Human PH domain affinity *in vitro* and localization *in vivo*

See above for color and font key (Sources for affinity and localization include Kavran *et al*, 1998; Ferguson *et al*, 2000; Snyder *et al*, 2001, and unpublished data). Localization data from mouse was used (See WEHI-231 and RAW 264.7 Image Data in http://www.signaling-gateway.org for details). * While not on the list of 66 representative PH domains, the PH domains of OSBP1 and FAPP1 are related to FAPP2. In particular, the PH domains of FAPP1 and FAPP2 have similar phosphoinositide- and Arf1-binding properties *in vivo* and *in vitro* (Godi *et al*, 2004).

Table 3 Mouse PH domain localization *in vivo*

Font: Black for PH domains represented in original proposal, Violet for alternate PH domains in the same phylogenetic class. Note that each PH domain listed represents a single phylogenetic class. (See WEHI-231 and RAW 264.7 Image Data in http://www.signaling-gateway.org for details).

A



Key

PI3P	PI(3,5)P2	PI(3,4,5)P3
1	2	3
PI4P	PI(4,5)P2	
4	5	
PI5P	PI(3,4)P2	
6	7	
PC	PI	PS
8	9	10
PG	PA	DAG
11	12	13

Fig.1A SH3BP-2 PH domain is a moderate affinity, PI(3,4)P2-specific PH domain

Protein-Lipid Overlay Assay protocol detailed in Yu *et al*, 2004 The phospholipids are spotted at the positions indicated on the key. The intensity of the spots are indicative of radiolabeled protein bound to lipid. GST-PH domain fusion of SH3BP-2 shows selectivity for PI(3,4)P2

B

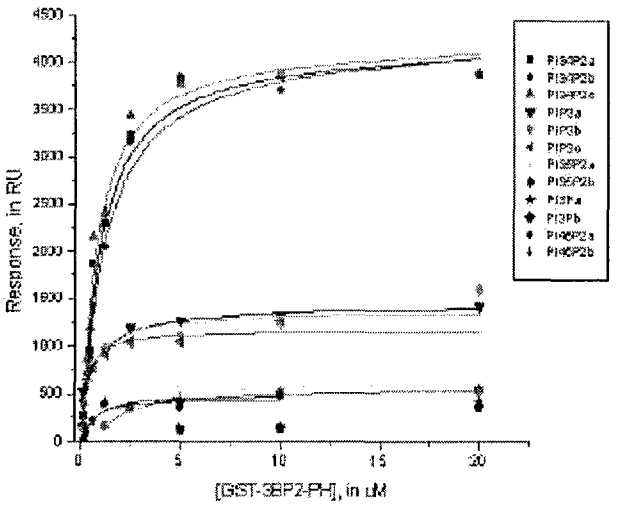


Fig.1B SH3BP-2 PH domain is a moderate affinity, PI(3,4)P2-specific PH domain

SPR Binding Assay protocol detailed in Yu *et al*, 2004 BIAcore response is based on refractive index changes that accompany protein binding to a lipid-coated chip surface. The apparent Kd is calculated from repeated iterations of $Y=(R_{max}*[(1/Kd)*X]/(1+((1/Kd)*X)))+ff$, where R_{max} is the maximal response and ff is the correction factor

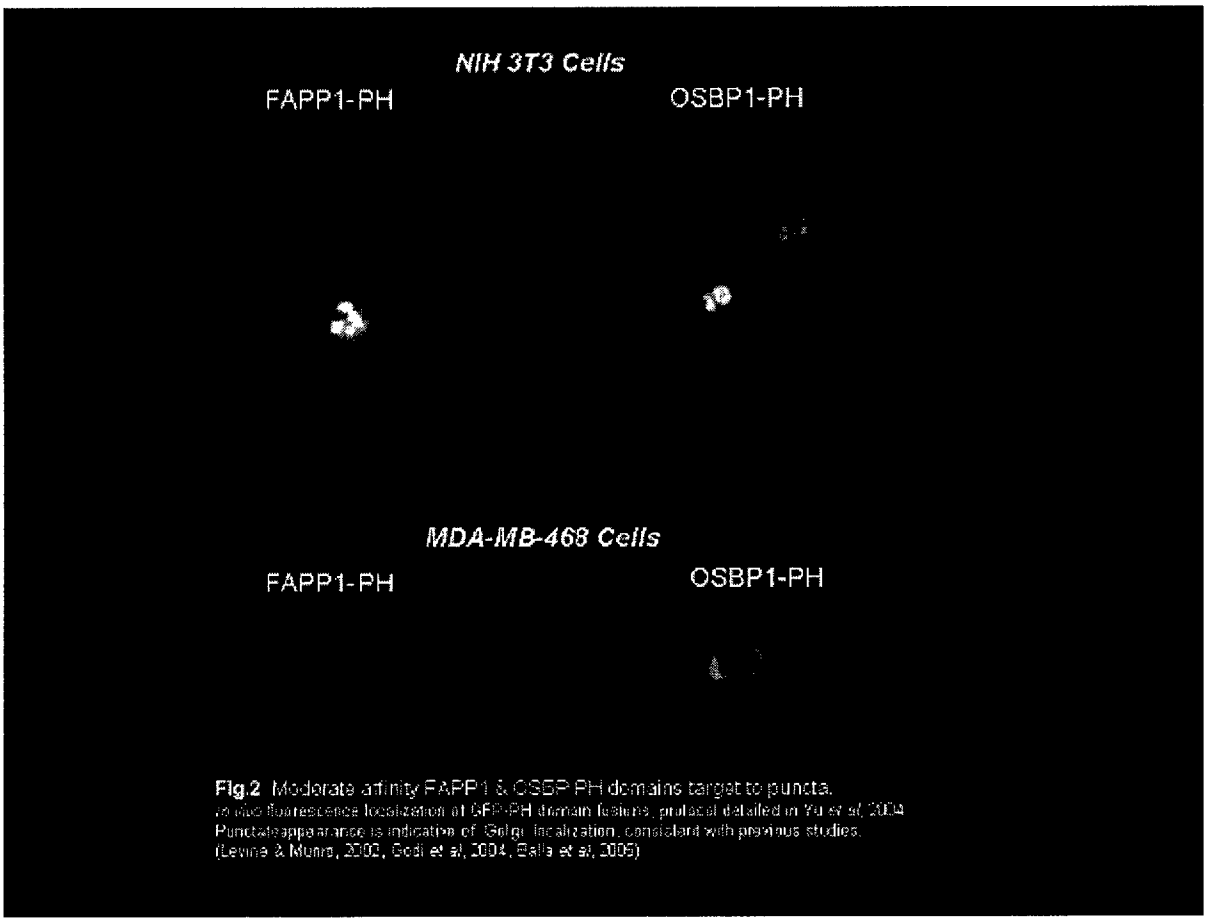


Fig.2 Moderate affinity FAPP1 & OSBP PH domains target to puncta.
In vivo fluorescence localization of GFP-PH domain fusions, protocol detailed in Yu *et al*, 2004. Punctate appearance is indicative of Golgi localization, consistent with previous studies. (Levine & Munro, 2002, Godi *et al*, 2004, Balla *et al*, 2005)

FAPP1 & OSBP PH domains are promiscuous for phosphoinositides *in vitro*.

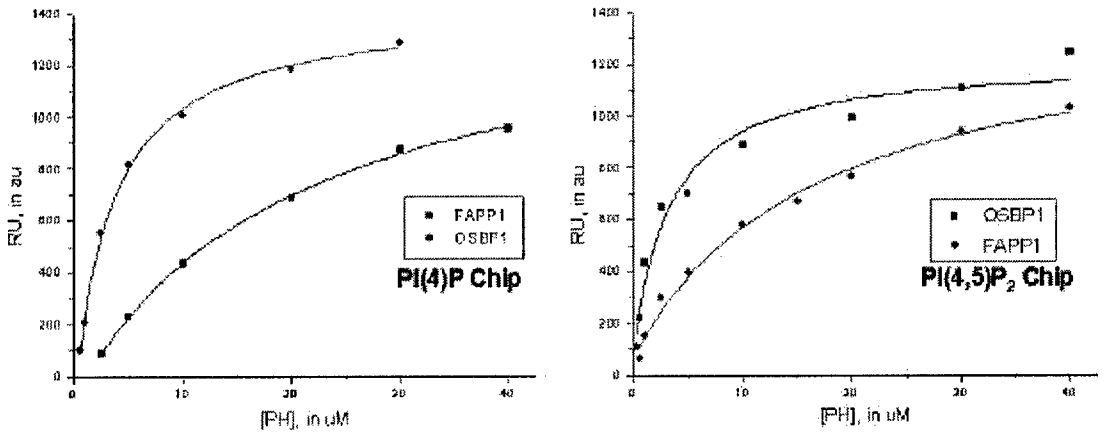


Fig.3 FAPP1 & OSBP PH domains are promiscuous for phosphoinositides *in vitro*.
SPR Binding Assay protocol detailed in Yu *et al*, 2004.
BIAcore response is based on refractive index changes that accompany protein binding to a lipid-coated chip surface. The apparent Kd is calculated from repeated iterations of:
Y=(Rmax*((1/Kd)*X))/(1+((1/Kd)*X)))+cf, where Rmax is the maximal response and cf is the correction factor. The Kds of GST-PH domain fusion of OSBP1 and FAPP1 for PI(4)P are 3.5 and 21 nM, and for PI(4,5)P2 are 3.3 and 17 nM, respectively. Thus, there is little or no selectivity of these PH domains for PI(4)P over PI(4,5)P2.

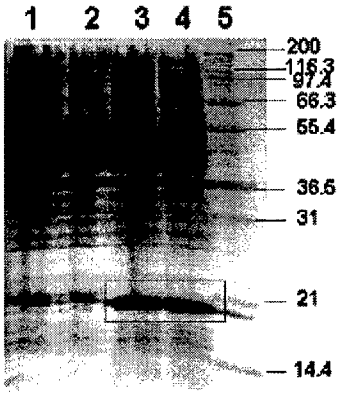


Fig.4 15% SDS-PAGE of myristoylated small GTPase Arf1 expressed in E. coli
One wild-type and one yeast-human chimeric construct obtained from Paul Randazzo (NIH)
Protocols for expression and purification obtained in Manser & Leung (eds), 2002.
Lanes 1 and 3 are wild-type myrArf1, lanes 2 and 4 are a yeast-human chimera ([LFASK]-myrArf1)
Lanes 1 and 2 are uninduced, lanes 3 and 4 are induced with 1 mM IPTG.
Lane 5 is an Mark 12 protein ladder (Invitrogen); the Mol. Wt. in Kb are listed on the right.
Myristoylated Arf1 is highlighted in red.

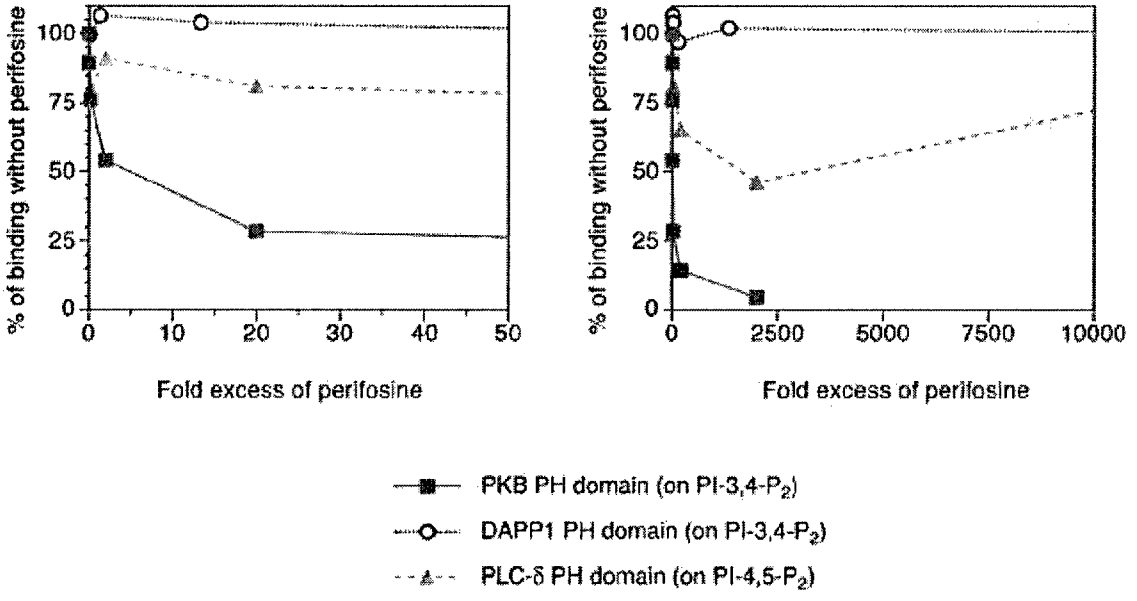


Fig.5 Perifosine selectively inhibits binding of PKB PH domain to PtdIns(3,4)P_2

SPR Binding Assay protocol to determine K_d as described in Yu *et al*, 2004. K_d concentrations used for perifosine competition experiments (PKB, 0.8 μM; DAPP1, 0.1 μM; PLCδ, 0.3 μM). PH domains incubated with perifosine for 30 min at RT before flowed over phosphoinositide-coated L1 chip. Increasing concentration of perifosine (x-axis) plotted versus RUs with perifosine addition, normalized against PH domain without perifosine (y-axis). DAPP1 PH domain is closest sequence-relative of PKB, and has essentially identical phosphoinositide binding specificity and affinity.

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Genome-Wide Analysis of Membrane Targeting by *S. cerevisiae* Pleckstrin Homology Domains

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Summary

Pleckstrin homology (PH) domains are small protein modules known for their ability to bind phosphoinositides and to drive membrane recruitment of their host proteins. We investigated phosphoinositide binding (in vitro and in vivo) and subcellular localization, and we modeled the electrostatic properties for all 33 PH domains encoded in the *S. cerevisiae* genome. Only one PH domain (from Num1p) binds phosphoinositides with high affinity and specificity. Six bind phosphoinositides with moderate affinity and little specificity and are membrane targeted in a phosphoinositide-dependent manner. Although all of the remaining 26 yeast PH domains bind phosphoinositides very weakly or not at all, three were nonetheless efficiently membrane targeted. Our proteome-wide analysis argues that membrane targeting is important for only ~30% of yeast PH domains and is defined by binding to both phosphoinositides and other targets. These findings have significant implications for understanding the function of proteins that contain this common domain.

Introduction

Pleckstrin homology (PH) domains are common modules of ~120 aa found in proteins involved in signaling, cytoskeletal organization, membrane transport, and modification of phospholipids. The core PH domain fold consists of a seven-stranded β sandwich capped off by a characteristic C-terminal α-helix and is also seen in several domain classes, including phosphotyrosine binding (PTB) domains, Ena/Vasp homology (EVH-1) domains, and a Ran binding domain (Blomberg et al., 1999; Lemmon and Ferguson, 2000). The PH domain fold appears to represent a structural module adaptable to several different binding functions, interacting with phosphoinositides in some cases and with protein targets in others (Lemmon and Ferguson, 2000).

The PH domain is the 11th most common domain in humans, with ~252 examples (International Human Genome Consortium, 2001). Despite this prevalence and the fact that 14 PH domain structures have been determined, the majority of PH domains are poorly understood, and it is not known how their functions vary across the genome. PH domains are best known for their ability to bind phosphoinositides and to be targeted to cellular membranes. For example, the PH domain from phospholipase C-δ_1 (PLCδ-PH) binds with high affinity and specificity to PtdIns(4,5)P_2 and is now frequently used as a probe to localize this phosphoinositide in living cells (Balla et al., 2000). Several PH domains (e.g., from Grp1 and PKB) bind tightly and specifically to the products of agonist-dependent phosphoinositide 3-kinases and drive signal-dependent recruitment of their host proteins to the plasma membrane.

PH domains with specific phosphoinositide recognition properties have been well studied and can be identified based on a sequence motif in the β1/β2 loop between the first two strands of the β sandwich (Dowler et al., 2000; Isakoff et al., 1998). However, more than 80% of PH domains do not have this β1/β2 loop sequence motif (or related sequences), and many have been shown to bind phosphoinositides only weakly and with little specificity (Kavran et al., 1998; Rameh et al., 1997; Takeuchi et al., 1997). Since only a small fraction of PH domains has been analyzed, it remains possible that there are examples capable of specifically recognizing phosphoinositides in a structurally distinct way. Alternatively, PH domains that do not bind phosphoinositides may be membrane targeted by binding to other ligands. Some PH domains may not be membrane targeted at all.

To investigate these possibilities from a genomic perspective, we analyzed all 33 *S. cerevisiae* PH domains identified (in late 2001) by the SMART database (Schultz et al., 2000). We investigated phosphoinositide binding in vitro and in vivo, as well as membrane targeting by the isolated PH domains. We also analyzed the electrostatic properties of structural models of each PH domain. Our results provide a genome-wide view of PH domain function, suggest that several PH domains have more than one binding target in cellular membranes, and argue that the best-known characteristics of PH domains are in fact the least common.

Results and Discussion

In Vitro Phosphoinositide Binding Specificity

We first investigated the phosphoinositide binding specificity of each yeast PH domain, employing a semiquantitative lipid overlay approach that has been used extensively in initial characterization of membrane targeting domains (Dowler et al., 2000; Kavran et al., 1998). Using domain boundaries guided by earlier structural studies of PH domains, we could generate sufficient protein for

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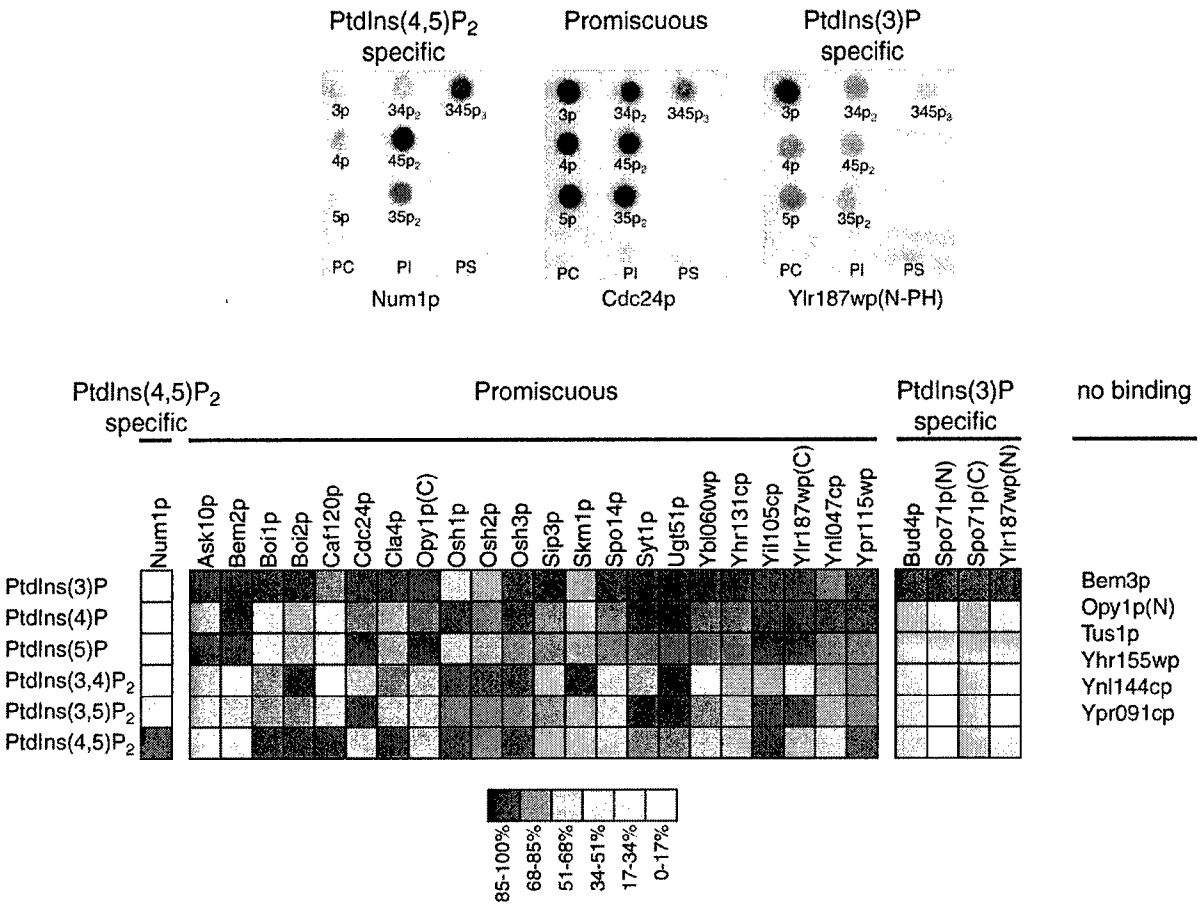

Figure 1. Phosphoinositide Binding Specificity of Yeast PH Domains Using a Lipid Overlay Method

In the upper panel, a representative lipid overlay experiment is shown for each specificity group (except the "no binding" group). In the lower panel, a semiquantitative representation of lipid overlay results for each PH domain is shown. The phosphoinositide that bound most strongly to each PH domain was arbitrarily scored as 100%. For a representative experiment, binding to other phosphoinositides is expressed as a percentage of this value and the boxes colored according to the legend. This scoring scheme does not allow comparison of affinities between PH domains, but color variation gives an impression of specificity.

this analysis for all 33 yeast PH domains (from 30 different proteins). Each PH domain was expressed as a GST fusion, labeled with ^{32}P, and used to probe nitrocellulose membranes bearing spots of relevant phosphoinositides. We anticipated that this analysis would identify *S. cerevisiae* PH domains with specific phosphoinositide targets and/or with novel specificities. Contrary to these expectations, the overwhelming impression was instead one of nonspecific, or promiscuous, phosphoinositide binding by PH domains (Figure 1). Some 67% of yeast PH domains (22 of 33) bound all phosphoinositides tested in this assay, with no clearly preferred binding partner, and six PH domains showed no detectable phosphoinositide binding. One PH domain (from Num1p) specifically recognized PtdIns(4,5)P_2 and four showed a preference for PtdIns(3)P, as reported for certain human and *Arabidopsis* PH domains (Dowler et al., 2000). In summary, our lipid overlay studies argue that specific phosphoinositide binding is a property of very few (<15%) yeast PH domains.

Only Seven Yeast PH Domains Bind Strongly to Phosphoinositides

Lipid overlay studies give an impression of phosphoinositide binding specificity but provide no useful information about binding affinities. To assess the strength of phospholipid binding by each yeast PH domain and to reassess specificity in a more physiological setting, we used surface plasmon resonance (SPR). Each GST/PH domain fusion (at ≥3 μM) was tested for binding to membranes containing phosphoinositides at 3% (mole/mole) in a phosphatidylcholine (DOPC) background (Figure 2). This was done for 27 PH domains (six failed to produce sufficiently well), using membranes with 3% PtdIns(4,5)P_2, PtdIns(3,5)P_2, PtdIns(3)P, or PtdIns(4)P. Binding to PtdIns(4,5)P_2 could be detected for just seven yeast PH domains (Figure 2A): those from Num1p, Boi1p, Boi2p, Cla4p, Osh1p, Osh2p, and Skm1p. In all other cases, SPR signals of less than 400 response units (RUs) were obtained, which are negligible compared with the 2000–4000 RUs measured for the seven strongly binding

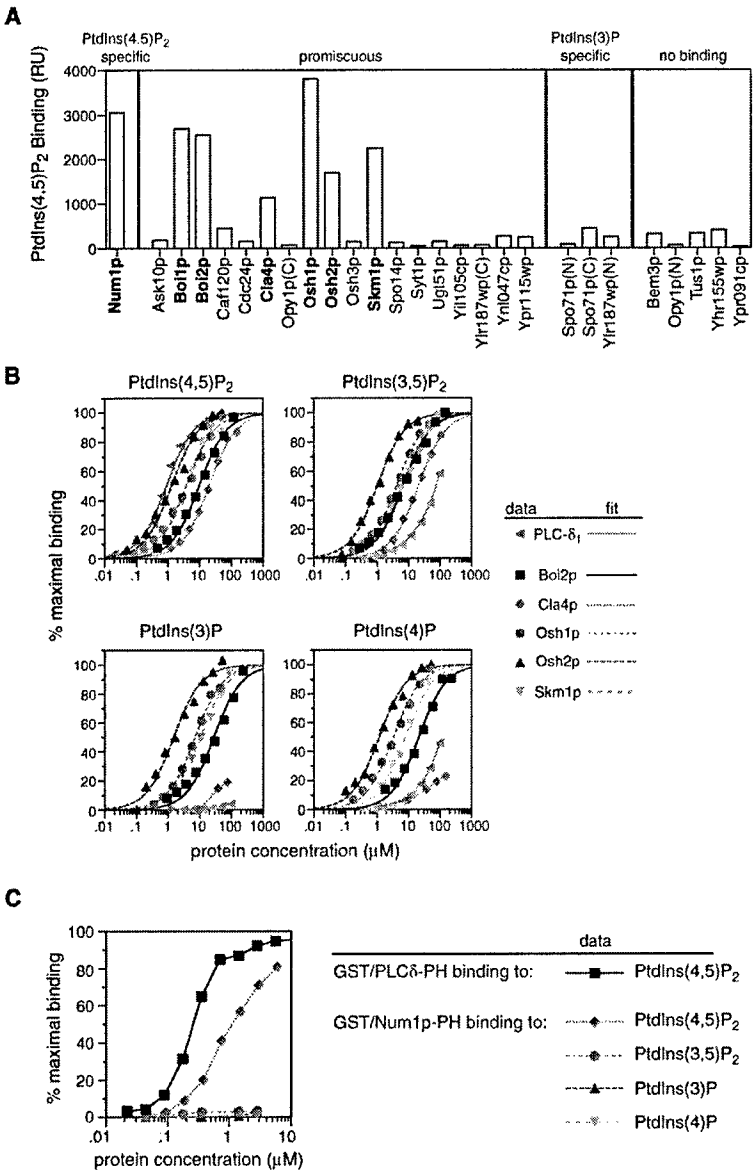

Figure 2. Surface Plasmon Resonance Analysis of Phosphoinositide Binding by Yeast PH Domains

(A) Each GST/PH fusion was flowed at ≥3 μM over a sensorchip containing 3% (mole/mole) PtdIns(4,5)P_2 in DOPC. PH domains that gave signals >1000 RUs are listed in bold type and were further analyzed. The PH domains from Bem2p, Bud4p, Sip3p, Ybl060wp, Yhr131cp, and Ynl144cp could not be produced in sufficient quantities for analysis with this approach.

(B) PH domains that bound significantly in (A) were subjected to more detailed binding analysis. Monomeric PH domains were flowed at a series of concentrations across surfaces containing 3% (mole/mole) PtdIns(4,5)P_2, PtdIns(3,5)P_2, PtdIns(3)P, or PtdIns(4)P. Binding signals are plotted against protein concentration, and the best-fit curves (with K_D values reported in Table 1) are superimposed. Data for PLCδ-PH are shown for comparison. Results are representative of at least three repeats, with errors given in Table 1.

(C) Phosphoinositide binding curves for the GST/Num1p-PH protein, as described in (B). Data for GST/PLCδ-PH are shown for comparison.

yeast PH domains or for a PLCδ-PH positive control. Essentially identical results were obtained with PtdIns(3,5)P_2, PtdIns(3)P, and PtdIns(4)P, although the Num1p and Cla4p PH domains (which show at least some specificity) gave weaker signals with other lipids (see below). The PH domains that appear PtdIns(3)P specific in Figure 1 did not bind detectably to this lipid in BIAcore studies, whereas positive-control PX and FYVE domains gave robust responses on the same sensorchip surfaces (data not shown).

Thus, only seven yeast PH domains bind phosphoinositides in vitro with micromolar or stronger K_D values as GST fusions. For the remainder that we tested, we estimate K_D values >20 μM, even with the avidity effect afforded by GST-mediated dimerization (Klein et al., 1998). In about half of the cases, additional experiments using 20–50 μM GST/PH fusion protein also gave no binding signal, placing K_D > 200–500 μM. Lipid overlay experiments nonetheless showed clear phosphoinositide binding for these cases, showing the sensitivity of this approach and illustrating that caution must be exercised in interpreting its results.

Most Yeast PH Domains that Bind Strongly to Phosphoinositides Show No Headgroup Specificity

We next quantitated phosphoinositide binding for the seven PH domains with positive signals in Figure 2A. To avoid well-documented avidity effects resulting from fusion to GST (Klein et al., 1998), we generated monomeric forms of each PH domain (except Num1p-PH and Boi1p-PH, which did not express well) as previously described (Ferguson et al., 1995). Figure 2B shows curves for binding of monomeric PH domains to each phosphoinositide found in yeast, with monomeric rat PLCδ-PH (Lemmon et al., 1995) as a comparative con-

Table 1. K_D Values for Phosphoinositide Binding by Monomeric Yeast PH Domains

PH Domain	PtdIns(4,5)P_2	PtdIns(3,5)P_2	PtdIns(3)P	PtdIns(4)P
Boi2p	9.7 ± 0.8 μM	6.6 μM	19.5 ± 11.7 μM	20.0 ± 1.3 μM
Cla4p	20.4 ± 6.4 μM	20.2 ± 0.4 μM	>100 μM	>100 μM
Osh1p	3.0 ± 1.0 μM	3.5 ± 0.8 μM	6.2 ± 1.3 μM	2.8 ± 0.8 μM
Osh2p	1.1 ± 0.3 μM	1.0 μM	1.5 ± 0.2 μM	1.3 ± 0.2 μM
Skm1p	3.9 ± 0.4 μM	6.4 μM	8.0 ± 3.7 μM	8.2 ± 0.4 μM
Rat PLC-δ_1	0.68 ± 0.28 μM	76.0 ± 4.7 μM	>100 μM	131 ± 19 μM

Data are from BIAcore experiments as shown in Figure 2B. Errors represent the standard deviation for K_D values obtained in at least three independent experiments.

trol. Best-fit K_D values are listed in Table 1. In agreement with our dot blot analyses, the Boi2p, Osh1p, Osh2p, and Skm1p PH domains showed little to no specificity, binding similarly to all four phosphoinositides. Equivalent binding characteristics were previously reported for the PH domain from OSBP, a mammalian homolog of Osh1p and Osh2p (Levine and Munro, 2002), and for Boi1p-PH (Hallett et al., 2002). Cla4p-PH showed a distinct preference for bisphosphorylated over monophosphorylated lipids, binding PtdIns(4,5)P_2 and PtdIns(3,5)P_2 with K_D values of ~20 μM but significantly less well to PtdIns(3)P and PtdIns(4)P (K_D > 100 μM). Parallel control studies using the same membrane surfaces confirmed the >100-fold preference of PLCδ-PH for PtdIns(4,5)P_2 over other phosphoinositides.

Num1p-PH Is the Only Yeast PH Domain that Binds Phosphoinositides Specifically and Strongly

Num1p-PH is the only PH domain that showed specificity in overlay studies (Figure 1) and measurable binding in Figure 2A. To analyze phosphoinositide binding by Num1p-PH in more detail, we used the highly expressed GST/Num1p-PH fusion because we could not produce large quantities of monomeric Num1p-PH. GST/Num1p-PH bound PtdIns(4,5)P_2 with an apparent K_D of ~1 μM, compared with ~0.25 μM measured for GST/PLCδ-PH (Figure 2C). By contrast with the five yeast PH domains analyzed in Figure 2B, Num1p-PH did not bind detectably to PtdIns(3,5)P_2, PtdIns(3)P, or PtdIns(4)P. Thus, Num1p-PH is a high-affinity PtdIns(4,5)P_2-specific PH domain that resembles PLCδ-PH in its phosphoinositide recognition properties. Our data suggest that Num1p-PH is the *only* PH domain in *S. cerevisiae* capable of specific and high-affinity phosphoinositide binding.

Only Six Yeast PH Domains Are Strongly Plasma Membrane Targeted, and Just Three of These Exhibit High-Affinity Phosphoinositide Binding

We were next interested to correlate in vitro phosphoinositide binding properties with subcellular localization of isolated yeast PH domains. With PH domains covering a wide range of phosphoinositide binding strengths (Table 1), we anticipated that this should provide insight into the affinity requirements for phosphoinositide-dependent membrane targeting in vivo, particularly given concerns that phosphoinositides may not be the only cellular targets of PH domains (Balla et al., 2000; Levine and Munro, 2002). We generated enhanced green fluorescent protein (EGFP) fusions of each isolated *S. cerevisiae* PH domain and investigated bulk localization in HeLa and yeast cells. Of the 33 EGFP/PH fusions, 21 showed only featureless and diffuse localization in HeLa and yeast cells. This group included all six PH domains that did not bind phosphoinositides in lipid overlay experiments. Six PH domains showed some nuclear localization, the relevance of which we have not yet established.

Of most interest are the six PH domains that show significant plasma membrane (PM) localization in both yeast and HeLa cells (from Cla4p, Num1p, Opy1p-C, Skm1p, Yil105cp, and Ynl047cp; Figure 3), several appearing to be enriched in patches at the PM (e.g., Yil105cp-PH and Opy1p C-PH). In addition, the Osh1p and Osh2p PH domains are localized to intracellular structures (Figure 3), identified as Golgi in previous studies of these two PH domains (Levine and Munro, 2001). Both of the Golgi-localized PH domains bind phosphoinositides strongly in vitro. However, this was true for only three of the six PM-localized PH domains (Num1p-PH, Cla4p-PH, and Skm1p-PH). The remaining three (Opy1p C-PH, Yil105cp-PH, and Ynl047cp-PH) bind phosphoinositides too weakly to be detectable using SPR, yet their PM fluorescence in yeast cells is the most intense (and is stronger than for PLCδ-PH). Thus, high-affinity phosphoinositide binding in vitro is not required for in vivo membrane targeting, suggesting that there are additional (or alternative) binding targets in the membrane.

Since analysis of EGFP fusion proteins will miss membrane recruitment mediated by low-abundance targets, we also used a more sensitive Ras recruitment assay (Isakoff et al., 1998). PH domains fused to a constitutively active Ha-Ras mutant can target active Ras to the membrane and thus rescue growth of *cdc25*^{ts} yeast. As shown in Figure 4A, PLCδ-PH rescues *cdc25*^{ts} yeast growth at 37°C by targeting the Ras fusion to PtdIns(4,5)P_2 in the yeast PM, whereas the dynamin PH domain does not. All six PH domains that were PM targeted as EGFP/PH fusions could recruit Ras to the membrane efficiently in this assay (Figure 4A). The Golgi-localized Osh1p and Osh2p PH domains also drove robust Ras recruitment, suggesting either partial PM localization (as in HeLa cells; Figure 3) or the ability of activated Ras to signal from internal membranes (Chiu et al., 2002). Of 11 additional PH domains analyzed with this approach (Figure 4A), 7 could not drive membrane recruitment of Ras, consistent with their diffuse cytoplasmic localization as EGFP/PH fusions. Boi2p-PH, which was not membrane targeted as an EGFP fusion protein [but binds PtdIns(4,5)P_2 with K_D ~10 μM], did promote membrane recruitment of Ras, as did Caf120p-PH (albeit weakly).

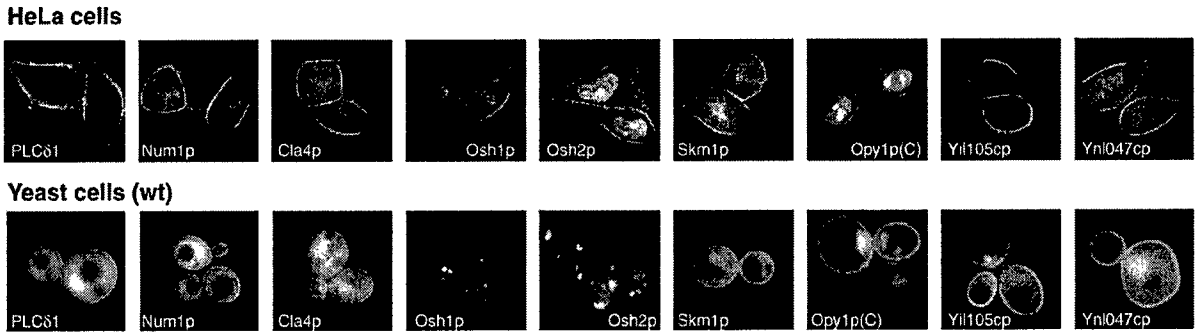

Figure 3. Subcellular Targeting of EGFP-Fused Yeast PH Domains in HeLa and Yeast Cells

Fluorescence micrographs are shown only for cases in which significant targeting was observed. Results for all other PH domains in yeast are given in Table 2.

Ask10p-PH and Ybl060wp-PH, which were both seen in the nucleus as EGFP fusions, could also target Ras to yeast membranes. Ras rescue studies thus confirmed all of the observations made with EGFP fusions but also suggested that many yeast PH domains (at least seven) have no significant membrane-targeting ability. The origin of the rescue seen with the Ask10p, Ybl060wp, and Caf120p PH domains is not yet clear and may result

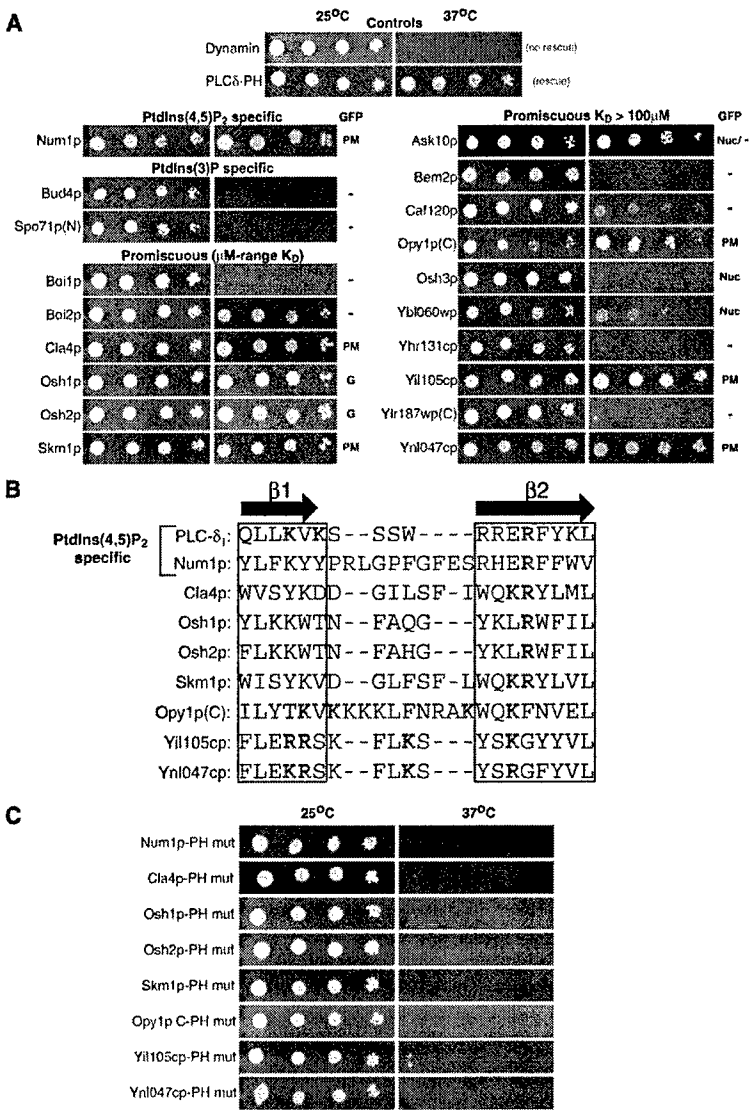

Figure 4. Membrane Targeting of Wild-Type and Mutated Yeast PH Domains Assessed by Ras Rescue

(A) Serial dilutions of *cdc25*^{ts} yeast cultures expressing the noted PH domain/RasQ61L(Δf) fusion were spotted onto duplicate selection plates lacking leucine and incubated at the permissive temperature (25°C) or restrictive temperature (37°C) for 7 to 8 days. Membrane targeting by the fused PH domain is required for yeast growth at 37°C (Isakoff et al., 1998). At the right of each experiment it is noted whether the EGFP/PH fusion was localized in yeast cells to the plasma membrane (PM), Golgi (G), nucleus (Nuc), or only the cytoplasm (–).

(B) Sequence alignment of the β1/β2 loop region of yeast PH domains that were membrane targeted as EGFP fusion proteins. The basic residues colored red [critical for PtdIns(4,5)P_2 binding in the case of PLCδ-PH] were simultaneously mutated to alanine to disrupt each predicted phosphoinositide binding site.

(C) Ras rescue experiments show that mutation of the presumed phosphoinositide binding site abolishes the ability of all eight PH domains to drive membrane recruitment. Western blot controls (data not shown) confirmed that Ras/PH fusion expression was not impaired by these mutations.

either from very weak phosphoinositide binding or from recognition of low-abundance membrane targets, both of which would be missed in EGFP fusion studies.

Membrane Targeting by PH Domains Is Impaired by Mutations in the Putative Phosphoinositide Binding Site

Every PH domain that was membrane targeted as an EGFP or Ras fusion also bound phosphoinositides in one of our assays (Table 2). We therefore asked whether mutations that abolish phosphoinositide binding also prevent membrane targeting in all cases. We focused on the eight PH domains targeted to the PM or elsewhere as EGFP fusion proteins. These are listed in Figure 4B alongside the β1/β2 loop sequences likely to constitute their phosphoinositide binding sites (Lemmon and Ferguson, 2000). For each PH domain, we replaced the basic residues, colored red in Figure 4B, with alanine, guided by the location of positively charged side chains in the PLCδ-PH β1/β2 loop that contact the PtdIns(4,5)P_2 headgroup (Ferguson et al., 1995). These mutations abolished or greatly diminished phosphoinositide binding in each case (data not shown), as assessed using SPR (for Num1p, Cla4p, Osh1p, Osh2p, and Skm1p PH domains) or lipid overlay experiments (for the Opy1p, Yil105cp, and Ynl047cp PH domains). This impaired phosphoinositide binding correlated in all cases with loss of membrane targeting by the PH domain, as assessed by the Ras rescue assay (Figure 4C). Western blotting with antibodies against an HA-tag in each fusion protein (data not shown) confirmed that expression levels were not affected by the mutations, excluding trivial misfolding explanations for the lack of Ras rescue.

Phosphoinositides Are Important for Recruitment of All Membrane-Targeted Yeast PH Domains Except Opy1p C-PH

The fact that β1/β2 loop mutations impair membrane targeting is consistent with a role for phosphoinositide binding in localizing these PH domains but does not provide direct evidence. Other ligands that bind to the same site on the PH domain could instead be responsible. To distinguish between these possibilities, we analyzed the localization of each EGFP/PH fusion in several *S. cerevisiae* mutants with different well-characterized alterations in phosphoinositide levels (Odorizzi et al., 2000). Using this approach, we showed that phosphoinositides play a direct role in membrane targeting in vivo of all but one (Opy1p C-PH) of the eight PH domains listed in Figure 4B.

We first investigated the effects of reducing PtdIns(4,5)P_2 levels, using *mss4^{ts}* cells. Mss4p is the PtdIns(4)P 5-kinase responsible for all yeast PtdIns(4,5)P_2 production (Desrivieres et al., 1998; Homma et al., 1998). In *mss4^{ts}* cells (AAY202; Stefan et al., 2002) at the permissive temperature (26°C), PtdIns(4,5)P_2 levels are ~57% of those in wild-type cells and fall ~3-fold further (to <20% of wild-type levels) at the restrictive temperature (Stefan et al., 2002). Levels of PtdIns(4)P, PtdIns(3)P, and PtdIns(3,5)P_2 are unaffected. In *mss4^{ts}* cells, membrane localization of Num1p-PH was difficult to discern even at the permissive temperature (Figure 5), probably because of reduced PtdIns(4,5)P_2 levels (this was also true for monomeric EGFP/PLCδ-PH). However, PM localization of the PH domains from Cla4p, Skm1p, Opy1p-C, Yil105cp, and Ynl047cp was quite clear in *mss4^{ts}* cells at 26°C. When PtdIns(4,5)P_2 levels were further reduced by shifting to 37°C, PM localization of all but the Opy1p C-terminal PH domain was greatly reduced. This finding argues that PtdIns(4,5)P_2 contributes significantly to membrane targeting of the Cla4p, Skm1p, Yil105cp, and Ynl047cp PH domains. By contrast, PM localization of Opy1p C-PH appears to be PtdIns(4,5)P_2 independent as does the intracellular punctate localization of the Osh1p and Osh2p PH domains. Similar results were obtained in *stt4^{ts}* cells at the restrictive temperature (data not shown), where inactivation of the Stt4p PtdIns 4-kinase reduces PM levels of both PtdIns(4)P and PtdIns(4,5)P_2 (Audhya and Emr, 2002; Audhya et al., 2000).

The type III PtdIns 4-kinase Pik1p appears to synthesize a Golgi-located PtdIns(4)P pool that is required for normal secretion (Audhya et al., 2000; Hama et al., 1999; Walch-Solimena and Novick, 1999). Reducing Golgi PtdIns(4)P levels by shifting *pik1^{ts}* cells to 37°C did not affect localization of the Num1p, Cla4p, Skm1p, Opy1p, Yil105cp, or Ynl047cp PH domains (data not shown). However, as previously reported (Levine and Munro, 2002), the punctate intracellular localization of Osh1p-PH and Osh2p-PH (both found at the Golgi) was substantially diminished. In *stt4^{ts}/pik1^{ts}* double mutants (Audhya et al., 2000), only Opy1p C-PH remained significantly localized (although its expression appeared to be toxic).

Another approach to manipulating cellular PtdIns(4)P and PtdIns(4,5)P_2 levels is to use yeast with mutations in phosphoinositide phosphatases. For example, in *sjl1*Δ cells, which lack the synaptojanin family PtdIns(4,5)P_2 5-phosphatase Sjl1p/Inp51p, PtdIns(4,5)P_2 levels are elevated by approximately 2-fold (Stefan et al., 2002; Stolz et al., 1998). As predicted from the findings in *mss4^{ts}* cells, this PtdIns(4,5)P_2 accumulation results in enhanced PM localization of the Num1p, Cla4p, Skm1p, Yil105cp, and Ynl047cp PH domains in *sjl1*Δ cells (Figure 5). Localization of Opy1p C-PH was only slightly enhanced (if at all), and Osh1p-PH and Osh2p-PH were unaffected. To investigate the effect of accumulating PtdIns(4)P at the PM, we also analyzed PH domain localization in cells lacking the Sac1p phosphatase. In *sac1*Δ cells, PM PtdIns(4)P (generated by Stt4p) accumulates to levels 20-fold higher than normal (Foti et al., 2001). PtdIns(3)P and PtdIns(3,5)P_2 levels are also slightly elevated (by 2-fold), and PtdIns(4,5)P_2 levels are depressed by around 75% (Foti et al., 2001). We found that Num1p-PH and Cla4p-PH are entirely cytoplasmic in *sac1*Δ cells, consistent with the reduced PtdIns(4,5)P_2 levels. The Skm1p, Yil105cp, and Ynl047cp PH domains all showed reduced but nonetheless discernible levels of PM localization. This is consistent with the fact that they bind similarly to PtdIns(4,5)P_2 and PtdIns(4)P in vitro or with the possibility that they have additional binding targets. The most dramatic effect of deleting *SAC1* was to promote strong PM localization of the Osh2p PH domain (Figure 5). Rather surprisingly (but consistent with a previous report of Levine and Munro, 2002), Osh1p-PH was not seen at the PM in *sac1*Δ cells. It therefore appears that, while Osh2p-PH can bind PtdIns(4)P at both the

Table 2. Summary of Phosphoinositide Binding Properties of Yeast PH Domains and Their Localization in Wild-Type Yeast Cells

	Ask10p	Bem2p	Bem3p	Boi1p	Boi2p	Bud4p	Caf120p	Cdc24p	Cla4p	Num1p	Opy1p N
PM	−	−	−	−	−	−	−	−	+/−	+	−
Diffuse cytoplasm	+	+	+	+	+	+	+	+	+	−/+	+
Punctate cytoplasm	−	−	−	−	−	−	−	−	−	−	−
Nucleus[a]	−/+	−	−	−	−	−	−	−	−	−	−
PI binding/specificity[b,c]	+/none[c]	+/none	−	+++/none	+++/none	+/PI(3)P	+/none	+/none	+++/PIP_2[e]	+++/PI(4,5)P_2	−
Intact protein[d]	*cytoplasm*	*cytoplasm*, bud neck	*cytoplasm*, bud neck	*cytoplasm*, bud, bud neck, cell periphery	*cytoplasm*, bud, bud neck, punctate	*bud neck*	*cytoplasm*, bud, bud neck	*cytoplasm*, nucleus	*cytoplasm*, bud	*punctate* composite	*cytoplasm*

	Opy1p C	Osh1p	Osh2p	Osh3p	Sip3p	Skm1p	Spo14p	Spo71p N	Spo71p C	Syt1p	Tus1p
PM	+	−	+/−	−	−	+	−	−	−	−	−
Diffuse cytoplasm	−/+	−	−	−	+	+/−	+	+	+	+	+
Punctate cytoplasm	−	+ (Golgi)	+	−	−	+/−	−	−	−	−	−
Nucleus[a]	+	−	−	+	−	−	−	−	−	−	−
PI binding/specificity[b,c]	+/none	+++/none	+++/none	+/none	+/none	+++/none	+/none	+/PI(3)P	+/PI(3)P	+/none	−
Intact protein[d]	cytoplasm	unscored	punctate composite	ambiguous	ambiguous	ambiguous	cytoplasm	unscored	unscored	unscored	unscored

	Ugt51p	Ybl060wp	Yhr131cp	Yhr155wp	Yil105cp	Ylr187wp N	Ylr187wpC	Ynl047cp	Ynl144cp	Ypr091cp	Ypr115wp
PM	−	−	−	−	+	−	−	+	−	−	−
Diffuse cytoplasm	+	−/+	+	+	−/+	+	+	−/+	+	+	−/+
Punctate cytoplasm	−	−	−	−	−	−	−	−	−	−	−
Nucleus[a]	−	+	−	−	−	−	−	+	−	−	+
PI binding/specificity[b,c]	+/none	+/none	+/none	−	+/none	+/PI(3)P	+/none	+/none	−	−	+/none
Intact protein[d]	cytoplasm	cytoplasm, bud neck	cytoplasm	mitochondria	punctate composite	cytoplasm, bud, bud neck, cell periphery	cytoplasm, bud, bud neck, cell periphery	unscored	unscored	ER	cytoplasm

[a] Nuclear localization of EGFP/PH fusion proteins may be an experimental artifact (Balla et al., 2000), and we have not attempted to interpret this finding.
[b] "+++" represents SPR-detectable binding; "+" represents binding detectable in lipid overlays but not SPR; "−" represents no binding by any assay.
[c] Preferred lipids are listed (none means no specificity).
[d] Results for intact proteins are taken from Huh et al. (2003).
[e] Cla4p-PH binds equally well to PtdIns(4,5)P_2 and PtdIns(3,5)P_2.

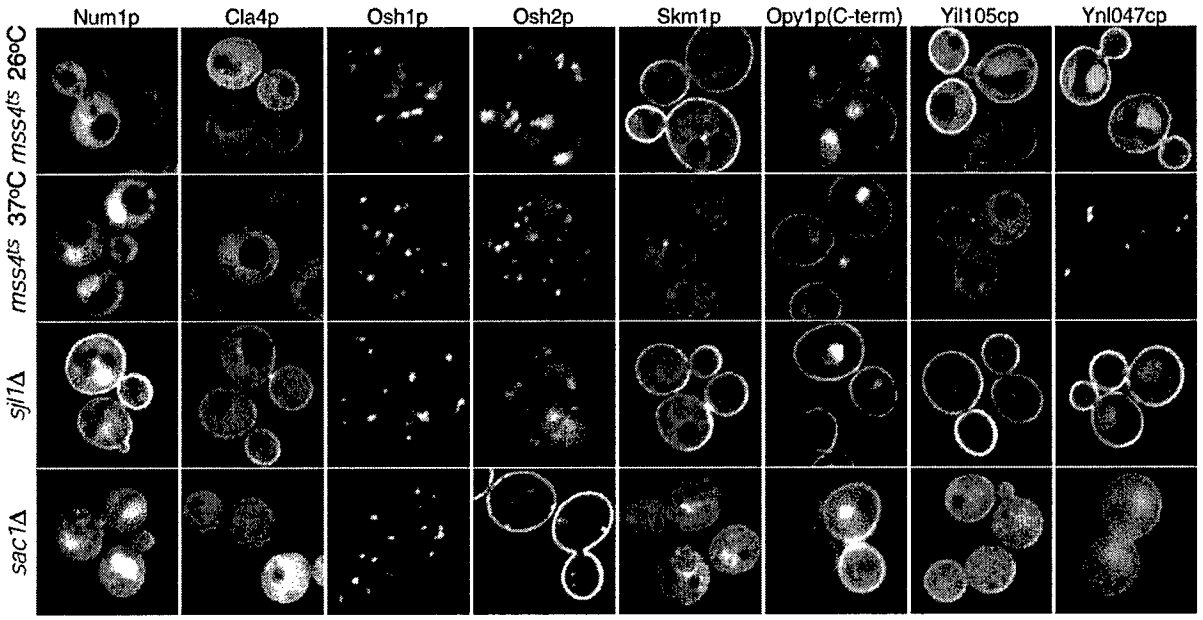

Figure 5. EGFP-PH Targeting in Yeast Cells with Altered PtdIns(4,5)P_2 or PtdIns(4)P Levels

Localization of the eight noted PH domains (fused to EGFP) was analyzed in *mss4*^{ts} cells [with reduced PtdIns(4,5)P_2 levels], grown to mid-log phase, and then incubated for 45 min at either 26°C or 37°C [where PtdIns(4,5)P_2 levels are further reduced], before being examined live by fluorescence microscopy. Localization of the same EGFP fusions in *sjl1*Δ cells [with elevated PM PtdIns(4,5)P_2] and *sac1*Δ cells [with elevated PM PtdIns(4)P] is also shown.

PM and the Golgi, Osh1p-PH recognizes PtdIns(4)P at the Golgi but not at the PM. As discussed below, this observation argues that factors in addition to phosphoinositides must influence the specificity of membrane targeting. Punctate intracellular localization was also seen in some yeast mutants for certain PH domains, such as Skm1p-PH and Ynl047cp-PH in *mss4*^{ts} cells at 37°C and Opy1p C-PH in wild-type cells (and for several cases in HeLa cells). We have not yet characterized these punctae, but the fact that they appear to differ for PH domains that have similar lipid binding specificities suggests that recruitment to them is defined by factors other than phosphoinositides.

These studies demonstrate a clear phosphoinositide dependence for PM targeting of the Num1p, Cla4p, Skm1p, Yil105cp, and Ynl047cp PH domains. For Num1p-PH and Cla4p-PH, PM localization correlates with PtdIns(4,5)P_2 levels in the membrane, consistent with their in vitro binding specificity. For the Skm1p, Yil105cp, and Ynl047cp PH domains, PM PtdIns(4)P may be able to substitute for this to some extent. Membrane localization of the Osh1p and Osh2p PH domains is also phosphoinositide dependent, and these PH domains appear to recognize distinct pools of PtdIns(4)P, in a way that cannot be explained by their phosphoinositide binding characteristics. Finally, membrane targeting of the Opy1p C-terminal PH domain is a clear exception, appearing phosphoinositide independent in these studies. As discussed in the conclusions, these findings argue that, while phosphoinositide binding clearly plays a role in many membrane targeting events observed here, in most cases additional binding targets must be invoked in the respective cellular membranes.

Sequence Characteristics of PH Domains with Different Phosphoinositide Binding and Membrane-Targeting Properties

We next asked whether the functional similarities of yeast PH domains with regards to membrane targeting and phosphoinositide binding are also reflected in their sequence relationships. The wide diversity of PH domain sequences makes this quite difficult, and no sequence patterns related to in vivo membrane targeting could be discerned. However, the genome-wide perspective showed that all PH domains with SPR-measurable phosphoinositide binding (from Num1p, Cla4p, Osh1p, Osh2p, Skm1p, Boi1p, and Boi2p) have a characteristic arrangement of basic residues in their β1/β2 loop (summarized in Figure 4B). This includes a conserved arginine in the middle of strand β2, known to be critical for PtdIns(4,5)P_2 binding by PLCδ-PH (Ferguson et al., 1995). In addition, most of the yeast PH domains that bind phosphoinositides with highest affinities (from Num1p, Osh1p, Osh2p, Boi1p, and Boi2p) have a characteristic lysine close to the end of strand β1, which also makes critical PtdIns(4,5)P_2 contacts in PLCδ-PH. Thus, all yeast PH domains with moderate to high in vitro phosphoinositide binding affinity possess a pattern of basic residues in their β1/β2 loop that resembles the well-characterized motif responsible for inositol phosphate headgroup recognition by PLCδ-PH and other mammalian PH domains (Lemmon and Ferguson, 2000). Only two other yeast PH domains have β1/β2 loop sequences that resemble this motif. One is Yhr131cp-PH, which we could not produce in sufficient quantities for SPR studies. The second is Osh3p-PH, which did not bind strongly to phosphoinositides (Figure 2A). Thus,

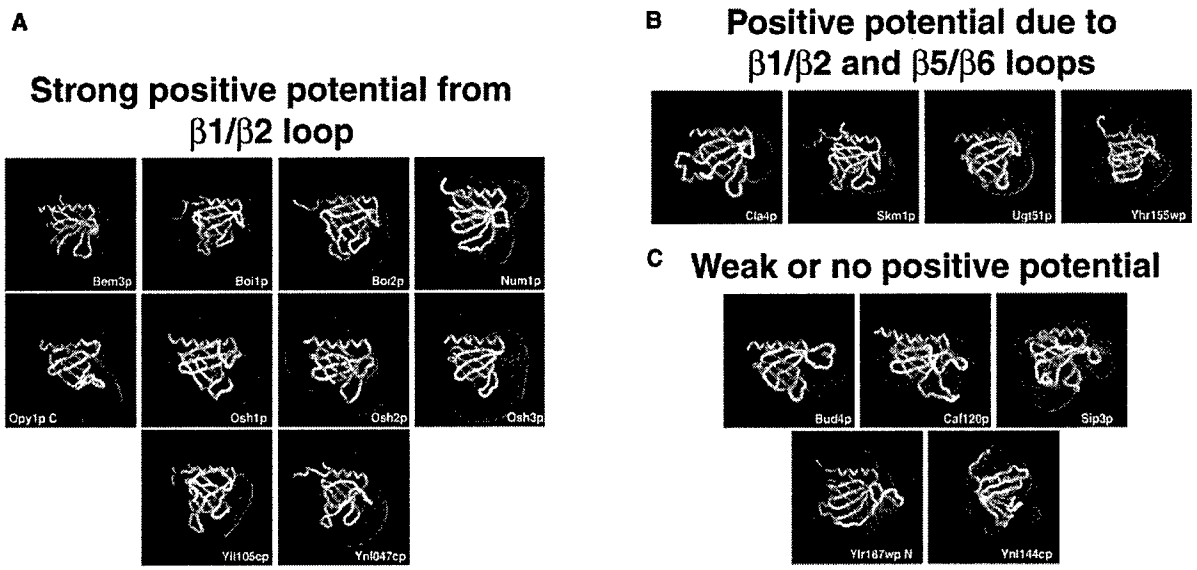

Figure 6. Electrostatic Characteristics of Modeled Yeast PH Domains

GRASP (Nicholls et al., 1991) representations are shown for the 19 PH domains for which reliable models could be obtained. Well-modeled PH domains were subdivided into three groups: those with strong positive potential arising from the β1/β2 loop (A), those with positive potential arising from the β1/β2 and β5/β6 loops (B), and those with little or no positive potential (C). Each PH domain model is shown in the same orientation, with the phosphoinositide binding side at the bottom of the panel. Alpha carbon traces are presented as white worms, and equipotential profiles are represented as blue (+25 mV) and red (−25 mV) meshes.

with Osh3p-PH as the only clear exception, the occurrence of this motif is a good predictor of moderate- to high-affinity phosphoinositide binding, and there do not appear to be alternative motifs for phosphoinositide binding (or membrane targeting) in yeast PH domains.

Molecular Modeling of PH Domains Suggests that Delocalized Electrostatic Interactions Play a Key Role in Membrane Targeting

Independently of our phosphoinositide binding and membrane-targeting studies, we also generated homology models of the three-dimensional structures of all *S. cerevisiae* PH domains, using approaches described previously (Singh and Murray, 2003). We could model 19 of the 33 PH domains well, but large insertions in the presumed loop regions prevented reliable modeling of the remaining 14. The 19 well-modeled cases include all yeast PH domains for which membrane targeting was observed and all 7 for which phosphoinositide binding was detectable with SPR. The calculated electrostatic potentials of the 19 reliably modeled PH domains, depicted as Cα worms, are illustrated in Figure 6; blue and red meshes represent positive and negative electrostatic potential, respectively. All models are shown in a similar orientation, with the predicted phosphoinositide binding site (including the β1/β2 loop) at the bottom of each panel. We found that 14 of these PH domains have a significantly positively charged region that coincides with the predicted phosphoinositide binding site. Many of these models exhibit the electrostatic sidedness seen in most known PH domain structures (Lemmon and Ferguson, 2000), although some others (such as Osh2p-PH) have an almost completely positive electrostatic profile. Ten of the models with significant positive potential have a highly basic β1/β2 loop (Figure 6A) and four have a basic region that is bracketed by both the β1/β2 and β5/β6 loops (Figure 6B), as observed in the structure of the β spectrin PH domain (Macias et al., 1994). The remaining five models display very weak or no positive potential (Figure 6C). Information on all of these models may be accessed at http://maat.med.cornell.edu/YEAST/Yeast_PH_domains.html.

Nine of the fourteen PH domains predicted to have positively charged ligand binding sites were targeted to the PM or Golgi when analyzed as EGFP or Ras fusion proteins (the Boi2p, Cla4p, Num1p, Opy1p C, Osh1p, Osh2p, Skm1p, Yil105cp, and Ynl047cp PH domains). Seven of the fourteen positive PH domains displayed significant phosphoinositide binding by SPR (including Boi1p-PH, which was not membrane targeted). Only four of the PH domains with strong positive potential (from Bem3p, Osh3p, Ugt51p, and Yhr155wp) failed to show either membrane localization or strong phosphoinositide binding (although Osh3p-PH was nuclear). By contrast, of the five models that do not exhibit significant positive potential, all were for PH domains that were not membrane targeted and did not bind phosphoinositides in SPR studies.

A good correlation therefore emerges between the electrostatic properties of yeast PH domain models and their membrane targeting and/or phosphoinositide binding characteristics. Based solely on their electrostatic properties, our models predicted that 14 yeast PH domains would bind membranes (and/or phosphoinositides). Of these 14, experimental studies independently demonstrated that 10 behave as predicted. Similarly, no membrane binding or targeting was detected for the five PH domains with weak or absent positive potential. As recently described for phospholipase C PH domains

(Singh and Murray, 2003), this analysis therefore appears to have significant predictive value. Equally important, the fact that electrostatic considerations provide such good predictive power argues that delocalized electrostatic attraction plays a critical role in PH domain-mediated membrane targeting events.

Conclusions

Our genome-wide analysis argues that high-affinity and specific phosphoinositide binding is not a common property of *S. cerevisiae* PH domains. In fact, yeast have only one PH domain (from Num1p) with these characteristics. Although we have yet to characterize PtdIns(4,5)P_2 recognition by the Num1p PH domain in structural detail, it appears to resemble PLCδ-PH and may be of value as an additional PtdIns(4,5)P_2 probe. From a functional perspective, the PH domain of full-length Num1p has been shown to be necessary for its localization to cortical patches in yeast cells (Farkasovsky and Kuntzel, 1995). Num1p at the cortex binds tubulin and dynein and is thought to serve as a cortical anchor for dynein as it drives nuclear migration through the bud neck during mitosis (Bloom, 2001).

Most yeast PH domains show no evidence for membrane targeting as isolated domains and bind phosphoinositides too weakly to be measurable using SPR (Table 2). A recent global analysis of yeast protein localization (Huh et al., 2003) suggests that this does not simply reflect our focus on isolated PH domains. Indeed, in that study, only 4 of the 22 intact proteins that contain these 23 PH domains were significantly localized. Seven were unscored or ambiguous in localization; eleven were cytoplasmic; one was at the bud neck; one was mitochondrial; one was at the endoplasmic reticulum; and one was in the cytoplasm, bud, bud neck, and periphery (Table 2). Thus, whether isolated PH domains or intact proteins are considered, bulk localization to cellular membranes does not appear to be an important property of most of these proteins. Elucidating PH domain function in this context will be an interesting challenge.

Two of the most striking conclusions of our study are: (1) that very similar degrees of membrane localization are seen for PH domains with very different phosphoinositide binding affinities (e.g., compare Yil105cp-PH and Num1p-PH) and (2) that quite different localization is seen for PH domains with very similar phosphoinositide binding specificities (e.g., compare Boi2p-PH, Osh1p-PH, and Skm1p-PH).

These conclusions suggest that, while phosphoinositide binding certainly contributes to membrane targeting of most of these PH domains, it does not specify their location. Levine and Munro showed that specific Golgi targeting of the OSBP PH domain (closely related to Osh1p) requires its simultaneous binding to both PtdIns(4)P and another factor (possibly Arf1p) at the Golgi (Levine and Munro, 2002). Our studies in *sac1*Δ yeast support this further. Although they have very similar phosphoinositide binding specificities and affinities, Osh1p-PH recognizes PtdIns(4)P only at the Golgi, whereas Osh2p-PH is recruited to PtdIns(4)P at the Golgi or the PM. In other words, the two PH domains appear to recognize the same lipid but in different contexts.

Among proteins with the PH domain "superfold" (Blomberg et al., 1999), the best-known PH domains (e.g., PLCδ-PH and Grp1-PH) may represent one extreme where the primary (or only) ligand is phosphoinositide. Num1p-PH is the only example of this sort among *S. cerevisiae* PH domains. At another extreme are the PTB, EVH1, and other domains with specific protein ligands that bind in distinct ways. Opy1p C-PH may resemble one of these examples and may have a protein rather than phosphoinositide target. Other yeast PH domains appear to lie between these extremes, perhaps being able to interact with both phosphoinositide *and* protein (or other target). Indeed, simultaneous binding of protein and phosphoinositide ligands to distinct sites has been reported for several domains with the PH domain fold. One example is the PH domain from β-adrenergic receptor kinase (βARK), which binds simultaneously to PtdIns(4,5)P_2 and $G_{\beta\gamma}$ subunits (Lodowski et al., 2003; Pitcher et al., 1995). Another example was provided by recent structural studies of the PTB domains from disabled-1 and disabled-2. The PH domain-like phosphoinositide binding site and PTB domain-like peptide binding site are *both* occupied simultaneously in crystal structures of these domains (Stolt et al., 2003; Yun et al., 2003).

The next phase in analyzing PH domain function in yeast (and humans) is to identify the proposed additional binding partners that define the specificity of membrane localization. In the meantime, the analysis of PH domain function presented here will help direct studies of both phosphoinositide signaling and PH domain-containing proteins in yeast, while also providing a framework for what to expect from the 250 or so examples in the human proteome.

Experimental Procedures

Production and Purification of GST/PH Fusion and Monomeric PH Proteins

To generate GST/PH fusions in *E. coli*, PH domain-encoding DNA fragments were PCR amplified from yeast genomic DNA and subcloned into pGEX-2TK (Amersham-Pharmacia) or pGSTag (Ron and Dressler, 1992). The PH domain boundaries were Ask10p(465-725), Bem2p(1787-1957), Bem3p(632-752), Boi1p(756-906), Boi2p(748-898), Bud4p(1158-1296), Caf120p(61-215), Cdc24p(465-678), Cla4p (58-193), Num1p(2563-2692), Opy1p-N(1-155), Opy1p-C(209-324), Osh1p(267-388), Osh2p(277-398), Osh3p(212-321), Sip3p(308-430), Skm1p(1-132), Spo14p(487-668), Spo71p-N(738-973), Spo71p-C(1022-1241), Syt1p(836-1074), Tus1p(703-883), Ugt51p(234-349), Ybl060wp(384-558), Yhr131cp(155-302), Yhr155wp(305-427), Yil105cp (452-588), Ylr187wp-N(76-232), Ylr187wp-C(243-447), Ynl047cp(429-562), Ynl144cp(170-312), Ypr091cp(109-273), and Ypr115wp(478-731). Site-directed mutagenesis used the QuikChange kit (Stratagene). GST/PH domain fusions were produced and purified as described (Klein et al., 1998).

For generation of untagged or hexahistidine-tagged PH domains in *E. coli*, the PH domains of Boi2p (amino acids 755-891), Cla4p (59-193), Osh1p (279-383), Osh2p (282-389), and Skm1p (1-132) were subcloned into pET11a (Cla4p), pET21a (Boi2p), or pET15b (Osh1p, Osh2p, and Skm1p). Cla4p-PH and PLCδ-PH were purified by cation exchange and gel filtration chromatography (Ferguson et al., 1995). Others were purified by Ni-NTA chromatography (Qiagen) followed by gel filtration.

Dot Blot Overlay Assay

Lipid overlay assays using ^{32}P-labeled GST/PH fusions were performed exactly as described (Kavran et al., 1998).

Surface Plasmon Resonance Analysis of Phosphoinositide Binding

Dioleoylphosphatidylcholine (DOPC) vesicles with or without 3% (mole/mole) added phosphoinositide were prepared and immobilized on L1 sensor chips, and binding experiments were performed as described (Erb et al., 2000; Yu and Lemmon, 2001). Binding to a reference DOPC-only surface was measured simultaneously and subtracted as background. For purified GST/PH fusions, protein concentration was determined by SDS-PAGE analysis and comparison with BSA standards. For quantitative analysis of binding by purified monomeric PH domains (Figure 2B), protein concentration was determined by absorbance at 280 nm using calculated extinction coefficients.

Microscopy

For analysis of PH domain localization in yeast, DNA fragments encoding each PH domain (with the same boundaries used for GST/PH fusions) were subcloned into pGO-GFP (Cowles et al., 1997). For yeast images in Figure 3, these plasmids were transformed into BY4742 using standard methods, cells were prepared as previously described (Audhya and Emr, 2002), and images were collected using a Leica-DMIRBE microscope at 100× magnification and processed using OpenLab deconvolution software (Improvision). For images in Figure 5, the relevant GFP fusion plasmids were transformed into the following strains: *mss4^{ts}*-AAY202 (Stefan et al., 2002), *sjl1*Δ-YCS62 (Stefan et al., 2002), *sac1*Δ-MFY62 (Foti et al., 2001), *stt4^{ts}*-AAY102 (Audhya et al., 2000), *pik1^{ts}*-AAY104 (Audhya et al., 2000), and *stt4^{ts}/pik1^{ts}* (Audhya et al., 2000). Cells were prepared at the permissive and restrictive temperatures as previously described (Audhya and Emr, 2002). Images were collected using a Zeiss Axiovert S1002TV microscope and processed with the Delta Vision deconvolution system.

For expression of EGFP/PH fusions in mammalian cells, PH domain-containing DNA fragments were subcloned into pEGFP-C1 or pEGFP-C3 (Clontech). HeLa cells grown on 35 mm glass bottom dishes (MatTek) were transfected with 5–10 μg of plasmid DNA by calcium phosphate precipitation and imaged 24 hr later using a Leica-DMIRBE microscope at 40× magnification and processed with OpenLab deconvolution software.

Ras Rescue Assay

To facilitate C-terminal fusion of PH domains to RasQ61L(Δf), lacking the farnesylation site, a linker with an internal XhoI site, HA tag, and in-frame stop codon was inserted between the BamHI and NotI sites of p3S0B-SRS (Isakoff et al., 1998) to generate p3S0BL2. PCR-amplified DNA for each PH domain (using the boundaries employed for the most soluble GST fusion) was then subcloned between the BamHI and XhoI sites of p3S0BL2. The resulting plasmids were transformed into *cdc25^{ts}* yeast and transformants grown to mid-log phase in SD (leu dropout) minimal media for several days at room temperature. Cultures were serially diluted (to OD_{600} = 1, 10^{-1}, 10^{-2}, 10^{-3}), and 3 μl of each dilution was spotted in duplicate onto SD (leu dropout) plates. Plates were incubated at the permissive (25°C) or restrictive (37°C) temperature and photographed after 7 to 8 days. Anti-HA immunoblotting confirmed expression of each RasQ61L(Δf)/PH fusion.

Modeling Analysis

Structural templates were chosen from the Protein Data Bank for each PH domain using protein fold recognition programs as described (Singh and Murray, 2003). The sequence to be modeled (the target sequence) and the sequence of its structural template were then aligned based on a combination of (1) the results of local and global (pairwise and multiple) alignment algorithms, (2) alignment of predicted secondary structure elements of the target with known secondary structure of the structural template, and (3) threading analysis.

Implementing these as detailed elsewhere (Singh and Murray, 2003), homology models were constructed by overlaying the target sequence on the template structure according to the optimized sequence alignment. Alignments were further manually edited to produce models that maximized the fitness scores obtained in structure evaluation programs. The illustrations in Figure 6 were generated using GRASP (Nicholls et al., 1991).

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17
PH Domains

Mark A. Lemmon, and David Keleti

17.1
Introduction

The pleckstrin homology (PH) domain was first defined in two notes that were published in *Cell* and *Nature* in 1993 [1, 2], and a 1993 article in *Trends in Biochemical Sciences* [3] suggested that this domain is "a common piece in the structural patchwork of signaling proteins". PH domains were identified as stretches of 100–120 amino acids found in many (then) recently identified signaling molecules, occurring twice in the platelet protein pleckstrin. It would certainly have surprised the authors of these publications to learn – as was possible after the sequencing of the human genome nine years later [4] – that the PH domain they defined is the eleventh-most-common domain type in the human proteome, with some 252 examples. As we discuss in this chapter, the abundance of PH domains probably reflects the fact that their defining sequence characteristics are associated with a particularly stable protein fold that can have several different functions. In other words, there are large numbers of domains that are structurally related to PH domains (which is what the sequence analysis actually defines), and we are now appreciating that the functions of PH domains thus defined may be quite diverse. Indeed, unlike in the SH2 and SH3 domains, for example, there are no identifiable sequence motifs or even completely conserved residues in PH domains.

The identification of PH domains followed hard on the heels of the discovery and analysis of protein target recognition by Src homology domains 2 and 3 (SH2 and SH3) and the resulting conceptual leap in our understanding of intracellular signal transduction [5, 6]. Naturally, it was suggested that the PH domain might represent another small protein module that drives specific protein–protein interactions in cellular signaling, and many laboratories embarked upon searches to identify PH domain targets, using approaches that had borne fruit with SH2 and SH3 domains. Within a year, papers were published indicating that the βγ subunits of heterotrimeric G proteins [7, 8] and protein kinase C isoforms [9] are among the binding partners for PH domains. Although these interactions may

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well be relevant in some instances – certainly $G_{\beta\gamma}$ interaction is important for the PH domain of the β-adrenergic receptor kinase (βARK) [10, 11] – a large amount of effort failed to identify a common protein or peptide target of the PH domain. It thus became apparent that the PH domain is likely to differ significantly from SH2, SH3, or many subsequently identified domains in its function in intermolecular interactions.

A major advance in understanding at least some PH domains came in 1994, when Fesik's laboratory showed that the most N-terminal of the two PH domains from pleckstrin itself can bind to the lipid phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P_2) [12]. This finding set the stage for a large number of subsequent studies that have established a role for PH domains in phosphoinositide-dependent recruitment of proteins to cellular membranes. There are well studied PH domains that specifically recognize PtdIns(4,5)P_2 and others that specifically recognize phosphoinositides that are phosphorylated at their 3-position and are therefore only recruited to membranes after signal-dependent activation of phosphatidylinositol 3-kinases [13]. In these instances, the biology and its structural basis are now quite well understood, and some PH domains are known to be membrane targeted in a signal-regulated manner. However, more recent studies indicate that relatively few of the 252 PH domains recognize phosphoinositides in this way. Most PH domains may combine nonspecific phosphoinositide binding and protein recognition to drive membrane targeting (or other events), and we still have a great deal to learn about how this is achieved (and what are the targets).

17.2
PH Domain Structure and Phosphoinositide Binding

Crystallographic or NMR structures have now been described for 16 different PH domains. For six of these, the crystal structure was determined in complex with a phosphoinositide headgroup [14–19]. Just one example of a PH domain–protein interaction – the PH domain-mediated interaction of βARK with $G_{\beta\gamma}$ – has been visualized crystallographically [11]. In this section we focus on phosphoinositide binding by PH domains and return to PH domain–protein interactions in Section 17.4.

17.2.1
Overall Structure – The PH Domain Fold

In all instances, the core structure of the PH domain is the same. It can be described as a β sandwich or a partly open β barrel having seven strands (Figure 17.1). Strands β1 through β4 form a β sheet that is almost orthogonal to a second sheet (containing strands β5 through β7). Both sheets have the topology of a β meander, and the contribution of strand β1 to both sheets gives the structure its opened-barrel appearance, as can be seen most clearly in Figure 17.1b. Because of their right-handed twist, the two β sheets in the sandwich contact one another closely at two

(close) corners (left and right in Figure 17.1a), but are splayed apart at the other two (splayed) corners [20] (top and bottom in Figures 17.1a and b). One splayed corner is capped by a C-terminal α helix (α1) found in all PH domains. The other is covered by the β1/β2, β3/β4, and β6/β7 loops of the PH domain, which are the most variable in length and sequence among different PH domains and have been termed variable loops 1 through 3 [21]. These features of the PH domain fold have also been observed in five other protein domain families that were not identified by sequence analysis [22]. Each of these domains is involved in directing protein–protein interactions. They are the phosphotyrosine binding (PTB) domain [23], a Ran-binding domain [24], the enabled/VASP homology 1 (EVH1) domain [25], the third subdomain of the FERM (band four-point-one, ezrin, radixin, moesin) domain [26, 27], and a domain from neurobeachin [28].

A particular characteristic of PH domains that was noted early on [21, 29] is that they are often electrostatically polarized. With one exception (the PH domain from *Caenorhabditis elegans* UNC-89 [30]), all PH domains with known structure have a large area of positive electrostatic potential that surrounds variable loops 1 through 3 (marked in Figure 17.1) and regions of negative potential on other parts of their surface. The presence of the three variable loops in a region of positive potential is consistent with their constituting the binding site for negatively charged ligands such as phosphoinositides.

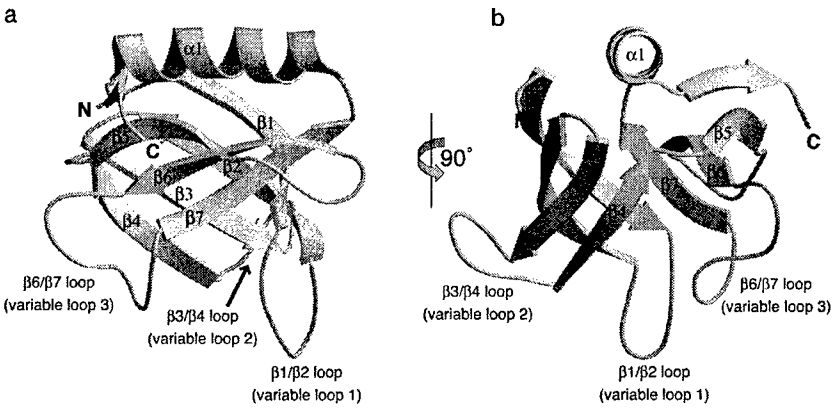

Figure 17.1 The PH domain fold. A ribbon representation of the PH domain from human dynamin-1 [21] is presented to illustrate the key points of the PH domain fold. The domain is shown from two orthogonal aspects, as indicated. Strands β1 through β7 are labeled, and the amphipathic C-terminal α helix that caps one splayed corner of the β sandwich is labeled α1. The N and C termini are marked, as are variable loops 1 to 3 that cap the splayed corner opposite α1. The variable loops correspond to the β1/β2, β3/β4, and β6/β7 loops, which are the most variable in length and sequence among PH domain sequences [21].

17.2.2 Structural Basis for Phosphoinositide Binding

Once phosphoinositides were identified as potential PH domain ligands [12], it became clear that certain PH domains specifically recognize a particular phosphoinositide (or subset of phosphoinositides) and bind with high affinity, but others bind more promiscuously and with much lower affinity. The PH domain from the N terminus of phospholipase C-δ_1 (PLC-δ_1) was the first shown to be capable of strong and specific phosphoinositide binding [31, 32]. The PLC-δ_1 PH domain binds to PtdIns(4,5)P_2 with a K_D in the 1–2 μM range, but at least 15-fold more weakly to any other phosphoinositide. By contrast, the N-terminal PH domain from pleckstrin and the β-spectrin PH domain are quite promiscuous in their phosphoinositide interactions and bind with K_D values in the 40 μM range [14, 33]. Structures of the PLC-δ_1 [15] and β-spectrin [14] PH domains with bound inositol-1,4,5-trisphosphate (Ins(1,4,5)P_3), the PtdIns(4,5)P_2 headgroup, provide insight into this specificity (or lack thereof).

17.2.2.1 High-affinity PtdIns(4,5)P_2 binding

When bound to the PLC-δ_1 PH domain [15], Ins(1,4,5)P_3 makes direct contact with the β1/β2 and β3/β4 loops (variable loops 1 and 2), as well as a water-mediated hydrogen bond to the β6/β7 loop (variable loop 3). The bound Ins(1,4,5)P_3 molecule is located in the center of the positively charged surface of the PH domain, suggesting a mode of association with phosphoinositide-containing membranes that is illustrated in Figure 17.2. Phosphoinositide binding is driven largely by interactions between phosphate groups of the Ins(1,4,5)P_3 headgroup and (mostly) basic sidechains in the β1/β2 loop region. As discussed in more detail below, it was subsequently found that all PH domains that recognize phosphoinositides with high affinity and specificity share a sequence motif (with variations for different phosphoinositides) in the β1/β2 loop region [34–36]. The PLC-δ_1 PH domain recognizes the spatial array of phosphate groups in Ins(1,4,5)P_3 through a stereochemical cooperativity of interactions between primarily basic sidechains and the phosphates. Inspection of the structure [15] makes it clear how this cooperativity would be disrupted by inositol phosphate isomers other than Ins(1,4,5)P_3, thus significantly reducing the affinity of binding.

17.2.2.2 Low-affinity PtdIns(4,5)P_2 Binding

The crystal structure of the complex between the β-spectrin PH domain and Ins(1,4,5)P_3 [14] paints a different picture with regard to specificity, although the bound headgroup is again found in the center of the positively charged face of the domain. Whereas Ins(1,4,5)P_3 projects into a clear binding pocket when it is bound to the PLC-δ_1 PH domain, it appears to lie on the surface of the PH domain in β-spectrin, with a few hydrogen bonds (7, compared with 12 in PLC-δ_1) between its phosphate groups and primarily surface-located β-spectrin sidechains. The lack of stereospecificity in inositol phosphate (and phosphoinositide) binding by the β-spectrin PH domain and others suggests that binding in these examples is driven

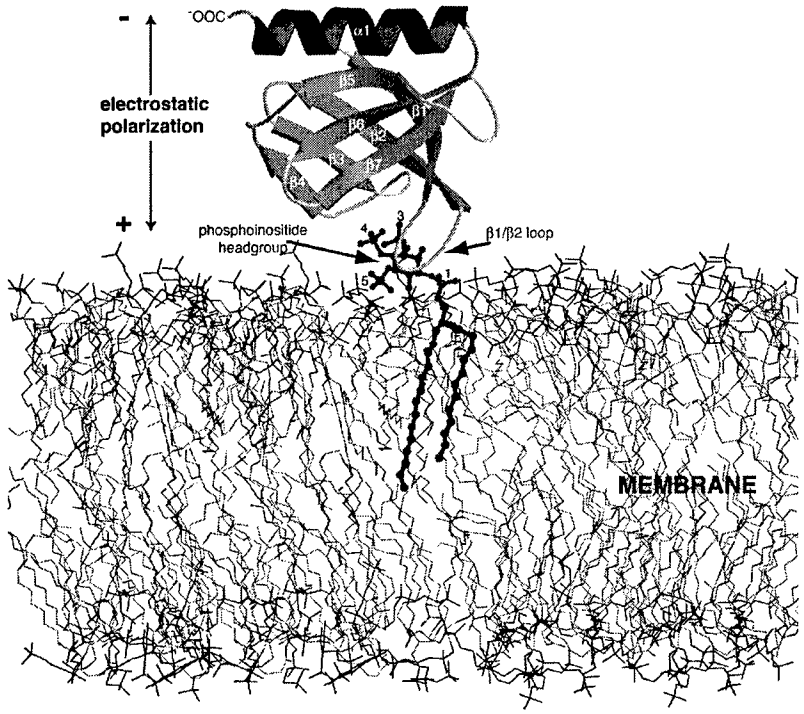

Figure 17.2 Hypothetical diagram of phosphoinositide-mediated membrane binding by a PH domain. The PH domain from DAPP1 [17] is used for illustration. This structure was determined in complex with Ins(1,3,4,5)P_4. Diacylglycerol has been added to the Ins(1,3,4,5)P_4 molecule to generate PtdIns(3,4,5)P_3, which has been placed in the context of a stick model of a phosphatidylcholine bilayer. The interaction and orientation of PtdIns(3,4,5)P_3 in the membrane is not intended to be accurate. This representation gives an impression of how the PH domain can interact with the phosphoinositide headgroup to drive its membrane association. The characteristic electrostatic polarization of PH domains, schematized to the left of DAPP1-PH, may also contribute to membrane association. The positively charged face (which includes the phosphoinositide-binding site) abuts the negatively charged membrane surface.

by delocalized electrostatic attraction between the positively charged surface of the PH domain and the highly negatively charged ligand. NMR studies of several PH domains in this class, including those from dynamin [37, 38], βARK [39], and pleckstrin-N [12], for example, also support this suggestion.

17.2.2.3 Specific Recognition of Phosphoinositide 3-kinase Products

One of the best-studied PH domain functions is specific recognition of phosphoinositides having a phosphate group at their 3 position. Almost all cell-surface agonists activate one or the other isoform of phosphoinositide 3-kinase (PI 3-kinase), leading to the phosphorylation of PtdIns(4,5)P_2 to yield PtdIns(3,4,5)P_3

[40]. PtdIns(4,5)P_2 is present constitutively in the plasma membrane of cells, and one estimate of its effective local concentration is approximately 5 mM [41]. By contrast, PtdIns(3,4,5)P_3 concentrations are estimated to be 1000 times lower (approximately 5 μM) prior to stimulation, rising to a maximum of around 200 μM following activation of PI 3-kinase by a cell-surface agonist [41]. A group of PH domains, including those from protein kinase B (PKB), Bruton's tyrosine kinase (Btk), and the general receptor for phosphoinositides-1 (Grp1), specifically recognize PtdIns(3,4,5)P_3 (or its immediate 5-dephosphorylation product PtdIns(3,4)P_2) and are directly recruited to the membrane as a result [13, 37, 42–45]. For these PH domains to be recruited to the plasma membrane only when PtdIns(3,4,5)P_3 is produced, their localization must be altered substantially by a local PtdIns(3,4,5)P_3 concentration of 200 μM, but not at all by a 25-fold local PtdIns(4,5)P_2 concentration of 5 mM. This feat is achieved with a selectivity for binding the PtdIns(3,4,5)P_3 headgroup (Ins(1,3,4,5)P_4) over the PtdIns(4,5)P_2 headgroup (Ins(1,4,5)P_3) of several hundred fold [13, 46], resulting from the addition of a single phosphate group.

Isakoff et al. [34] devised a convenient method for determining whether PH domains are capable of PtdIns(3,4,5)P_3-mediated (but not PtdIns(4,5)P_2-driven) membrane recruitment in yeast cells expressing a mammalian PI 3-kinase. Using

Figure 17.3 Sequence motif for high-affinity phosphoinositide binding to PH domains. (a) Sequences are shown for the β1 to β3 region of four PH domains specific for PI 3-kinase products and one (from PLC-δ_1) that is specific for PtdIns(4,5)P_2 as indicated. The positions of the lysine in strand β1, followed by the basic-X-basic pattern in strand β2 (see text), found in all PH domains that bind phosphoinositides with reasonably high affinity, are marked at the top. The motif used by Isakoff et al. [34] to predict which PH domains bind to PI 3-kinase products is shown below the sequences, and the residues found in Grp1-PH at each interacting position in the motif are marked. (b) Close-up of Ins(1,3,4,5)P_4 bound to the Grp1 PH domain [17]. Labels for residues in the sequence motif shown in panel A are boxed. The β1/β2 loop of Grp1-PH (variable loop 1) cradles the Ins(1,3,4,5)P_4 molecule, and motif residues fix the Ins(1,3,4,5)P_4 in position. Moving through the motif, the β1 leucine at the beginning of the motif projects into the hydrophobic core of the domain, helping to anchor the β1/β2 loop. The lysine in the middle of β1 (K273) forms hydrogen bonds with the both the 3- and 4-phosphate groups of the bound Ins(1,3,4,5)P_4. The amino acid immediately following strand β1 (G275) must have a small (or absent) sidechain to allow the orientation of the inositol ring shown here. R277 in the center of the β1/β2 loop closely approaches the 5-phosphate of the bound Ins(1,3,4,5)P_4 and is thought to contribute to PtdIns(3,4,5)P_3 specificity (this is absent from the PKB and DAPP1 PH domains). In strand β2, the first basic residue of the motif (K282 in Grp1-PH) makes a hydrogen bond with the 1-phosphate of Ins(1,3,4,5)P_4. The second (R284) hydrogen bonds extensively with the 3-phosphate. This conserved arginine corresponds to R40 in the PLC-δ_1 PH domain, R25 in PKB-PH, and R28 in Btk-PH (the site of XLA mutations). This interaction is critical for phosphoinositide binding. The aromatic sidechain at the end of β2 (F286 in Grp1-PH) anchors the β2 end of the β1/β2 loop into the hydrophobic core. Finally, the β3 tyrosine (Y295 in Grp1-PH) makes a hydrogen bond with the 4-phosphate of bound Ins(1,3,4,5)P_4. Specific to Grp1-PH, additional sidechains from a lysine, histidine, and asparagines (from the β6/β7 loop) interact with the 5-phosphate group to increase PtdIns(3,4,5)P_3 specificity.

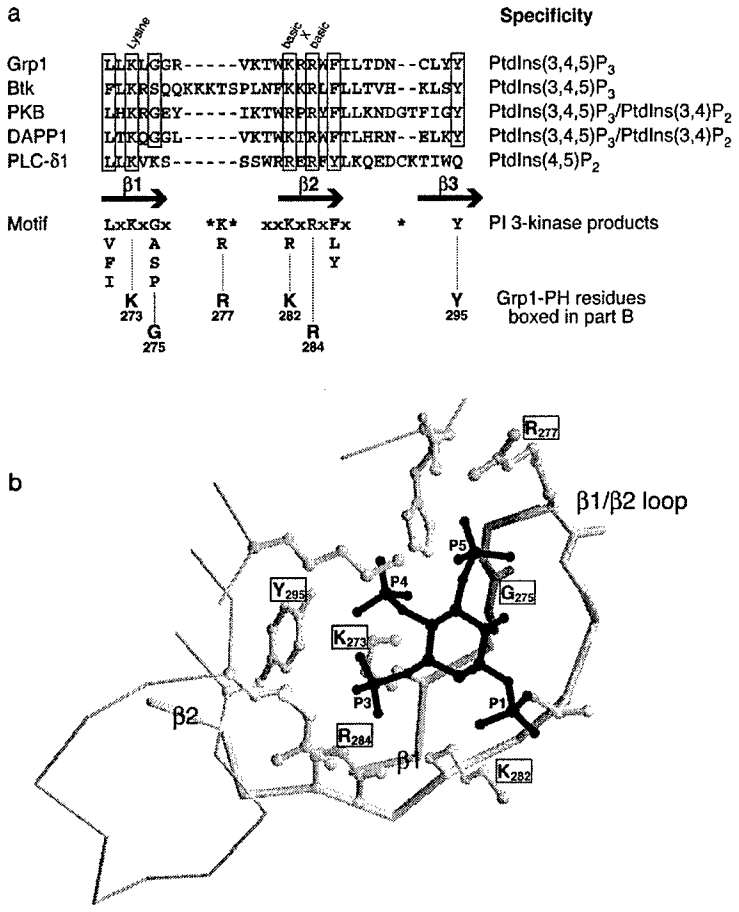

Figure 17.3 Sequence motif for high-affinity phosphoinositide binding to PH domains.

this approach they identified nearly all PH domains now known to have this property (and determined that many do not). Most interestingly, all PH domains that specifically recognize agonist-regulated PI 3-kinase products share a sequence motif in their β1/β2 loop region that is reminiscent of the corresponding region in the PLC-δ_1 PH domain. This motif is summarized in Figure 17.3a. Several subsequent X-ray crystal structures of PH domains bound to the headgroup of PtdIns(3,4,5)P_3 have provided a satisfying explanation of how this motif defines PH domain binding specificity [16–19]. The structural role of each conserved feature in the motif is illustrated in Figure 17.3b (and described in the figure legend).

The most critical elements of this motif are a lysine sidechain close to the end of strand β1 and two basic residues in the pattern 'basic-X-basic' at the beginning of

strand β2. The last of these basic residues corresponds to R28 in Btk, which is mutated in X-linked agammaglobulinemia (XLA) [47, 48], and is the 'standard' residue that is mutated to impair phosphoinositide binding by PH domains experimentally (R25 in the PKB PH domain, R284 in the Grp1 PH domain). As seen in Figure 17.3b, when Ins(1,3,4,5)P_4 is bound to the Grp1, Btk, DAPP1, or PKB PH domains, the β1 lysine sidechain (K273 in Grp1-PH) makes hydrogen bonds with both the 3- and 4-phosphates of Ins(1,3,4,5)P_4, and the basic residues in β2 (K282 and R284 in Grp1-PH) hydrogen bond with the 1- and 3-phosphates, respectively. These hydrogen bonds form the 'core' set of interactions and are supplemented by additional contacts mediated by the loop region and/or other parts of the PH domain that define the precise binding specificity. In Grp1-PH, for example, selectivity for PtdIns(3,4,5)P_3 over PtdIns(3,4)P_2 appears to be determined in part by R277 in the β1/β2 loop, which is close to the 5-phosphate group, plus sidechains of a unique β6/β7 insert that also hydrogen bond with this phosphate group [17]. The absence of corresponding basic residues in the PKB and DAPP1 PH domains explains their ability to bind PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 with very similar affinities.

Interestingly, the headgroups of PtdIns(3,4,5)P_3 or PtdIns(3,4)P_2 can be accommodated (with high affinity) only in the configuration shown in Figure 17.3b if the last residue in strand β1 has a small (or absent) sidechain (G275 in Grp1-PH). Accordingly, this position is occupied by glycine, serine, alanine, or proline in all PI 3-kinase product-specific PH domains. A larger sidechain at this position would clash with the inositol ring of the bound Ins(1,3,4,5)P_4 or Ins(1,3,4)P_3, requiring a reorientation that would disrupt the ability of the PH domain to maximize cooperativity between interactions involving the 1-, 3-, and 4-phosphates. Alteration of the backbone configuration in this region of the Grp1 PH domain by insertion of a glycine reduces its PtdIns(3,4,5)P_3-binding affinity and specificity [49] – actually enhancing its PtdIns(4,5)P_2-binding affinity.

The PLC-δ_1 PH domain maintains most features of the motif shown in Figure 17.3 but does not have a residue with a small sidechain at the end of strand β1. Possibly as a result of this difference, Ins(1,4,5)P_3 binds to the PLC-δ_1 PH domain in an orientation that is rotated by 180° (about an axis between the 1- and 4-phosphates) compared with that seen for Ins(1,3,4,5)P_4 in the Grp1-PH binding site in Figure 17.3b. In the complex between the PLC-δ_1 PH domain and Ins(1,4,5)P_3, the 1- and 4-phosphates of Ins(1,4,5)P_3 are similar in position to those of Ins(1,3,4,5)P_4 in Figure 17.3b. However, because of the ~180° flip, the position occupied by the 3-phosphate in Figure 17.3b is instead occupied by the 5-phosphate of Ins(1,4,5)P_3 in the PLC-δ_1 complex. Thus, the β1 lysine of the PLC-δ_1 PH domain interacts with the 4- and 5-phosphates of Ins(1,4,5)P_3 (rather than the 3- and 4-phosphates of Ins(1,3,4,5)P_4), and the two basic sidechains in strand β2 interact with the 1- and 5-phosphates instead of the 1- and 3-phosphates. Thus, the principles that guide specific recognition of PI 3-kinase products by the PKB, Grp1, Btk, and other PH domains are similar to those that direct specific PtdIns(4,5)P_2 recognition by the PLC-δ_1 PH domain. The distinct specificities arise from small differences in stereochemistry.

17.2.2.4 PH Domains with Other Phosphoinositide-binding Specificities

No PH domains have been identified that bind with high affinity and specificity to any phosphoinositide other than PtdIns(4,5)P_2, PtdIns(3,4,5)P_3, or PtdIns(3,4,5)P_3/PtdIns(3,4)P_2. Dowler et al. [35] searched EST databases for uncharacterized PH domains with β1/β2 loop sequences similar to the motif described above and analyzed their phosphoinositide-binding properties. One, which they named TAPP1 (for tandem PH domain-containing protein-1), has two PH domains, the most C-terminal of which contains the motif described above and appeared to be PtdIns(3,4)P_2-specific in their studies. Subsequent crystallographic and mutational analyses [50] indicated that this PH domain has an alanine (rather than glycine) in its β1/β2 loop, which disfavors accommodation of the 5-phosphate of PtdIns(3,4,5)P_3. However, although this PH domain does bind more strongly to PtdIns(3,4)P_2 than to PtdIns(3,4,5)P_3, its PtdIns(3,4,5)P_3 binding affinity is significant [17, 50]. In headgroup competition studies, TAPP1 C-PH shows only a ~five-fold preference for the PtdIns(3,4)P_2 headgroup over the PtdIns(3,4,5)P_3 headgroup (Sankaran, V. G. and M. A. L., unpublished data). It is therefore not clear to what extent TAPP1 is truly PtdIns(3,4)P_2-specific. The evidence that the C-terminal TAPP1 PH domain prefers PtdIns(3,4)P_2 is strong, and it has been demonstrated that TAPP1 does relocate to the plasma membrane of cells in response to agonists that promote PtdIns(3,4)P_2 production [51]. However, the in vitro binding properties of the C-terminal TAPP 1 PH domain argue that TAPP1 is also likely to be regulated by PtdIns(3,4,5)P_3 under most conditions.

Dowler et al. [35] also identified PH domains with motifs related to those described in Figure 17.3 that appeared to be specific for PtdIns(3)P (proteins named PEPP1 and AtPH1), PtdIns(3,5)P_2 (centaurin-β2), or PtdIns(4)P. The interactions observed in this study (using a lipid-overlay approach) with PtdIns(3)P and PtdIns(3,5)P_2 appear to have rather low affinity, and their physiological significance is not yet clear. In the FAPP1 PH domain, although the lipid-overlay studies of Dowler et al. [35] indicate PtdIns(4)P specificity, our own analysis with multiple approaches (D. K. and M. A. L., unpublished data), as well as published studies by Levine and Munro [52], suggest it is in fact quite promiscuous in its phosphoinositide binding. FAPP1-PH appears to bind with a K_D of around 10 μM to all phosphoinositides tested.

17.2.2.5 Sequence Predictors of Phosphoinositide Binding

In a recent genome-wide study of *Saccharomyces cerevisiae* PH domains [36], only seven (of 33 in the genome) were found to bind phosphoinositides sufficiently strongly for the interaction to be measured by standard techniques (i.e., K_D values less than approximately 50 μM). All of these PH domains have patterns of basic residues in their β1/β2 loop regions that resemble (or match) those seen in the PLC-δ_1 PH domain and the PH domains that specifically recognize PI 3-kinase products. It is therefore likely that the mode of phosphoinositide headgroup binding shown in Figure 17.3 represents a model that is relevant to all other PH domains that bind these ligands with significant affinity. At least in the *S. cerevisiae* genome there do not appear to be alternative modes of phosphoinositide recognition by PH domains. Moreover, with one exception in the yeast genome, the presence of a

lysine at the penultimate position in strand β1 plus the basic-X-basic pattern beginning at the second predicted residue of strand β2 are excellent predictors of significant phosphoinositide binding. Only one yeast PH domain having these features failed to bind phosphoinositides strongly in the studies of Yu et al [36]. Beyond these features, the characteristics described above and in the legend to Figure 17.3 can be used to predict whether the PH domain is likely to recognize PI 3-kinase products, and if so, whether it binds PtdIns(3,4,5)P_3 only or both PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3.

It is important to appreciate that the majority of PH domains do not contain sequence patterns of this type. In yeast, 25 of the 33 identifiable PH domains (~75%) cannot be predicted to bind strongly to phosphoinositides according to these criteria, and experimental studies show this to be true [36]. Assuming a similar distribution in humans, there are probably approximately 190 human PH domains that bind very weakly (if at all) to phosphoinositides. Developing an understanding of the function of these PH domains, and its structural basis, is an important challenge for the future.

17.3
Molecular and Signaling Function of PH Domains

As should be clear from Section 17.2, only a small minority of PH domains (perhaps 20%) bind strongly to phosphoinositides, and these are the most well understood structurally and functionally. Indeed, in the past few years PH domains have become best known as modules that specifically recognize phosphoinositides and thus drive membrane targeting of their host proteins. This membrane targeting can be recapitulated in vivo using the isolated PH domain and has been well studied. We discuss this group of PH domains in Section 17.3.1, separating them into three groups according to their phosphoinositide binding specificity. However, it is very important to realize that this is a property or function of only a minority of PH domains. To put this into one perspective, there is only a single PH domain capable of specific high-affinity phosphoinositide binding among all 33 PH domains found in *S. cerevisiae*. This is a PtdIns(4,5)P_2-specific PH domain from Num1p, a protein involved in nuclear migration [53]. Most PH domains (75% or more) do not bind strongly to phosphoinositides and, moreover, do not show any tendency to be targeted to cellular membranes when studied in isolation. The function of these PH domains is much more enigmatic and is considered in Sections 17.3.2 and 17.3.4.

17.3.1
PH Domains as Phosphoinositide-dependent Membrane-targeting Domains

The description of the structural properties of phosphoinositide-specific PH domains provides a good conceptual introduction to their function. In essence, they target the protein that contains them to membranes containing the phosphoinositide(s) to which they bind with high affinity. Whether their membrane targeting

is constitutive or signal-regulated depends on the phosphoinositide(s) that they recognize. Thus, PtdIns(4,5)P_2-specific PH domains are constitutively targeted to the plasma membrane and (to some extent) to other membranes, but PH domains that recognize only PI 3-kinase products remain cytoplasmic in unstimulated cells but are rapidly recruited to the plasma membrane upon activation of cell surface receptors. The clarity of our current view of phosphoinositide recognition by this class of PH domains has led to their use as in vivo probes of phosphoinositide production and location [54, 55]. The PLC-δ_1 PH domain fused to green fluorescent protein (GFP) has become an almost standard tool for observing the subcellular localization of PtdIns(4,5)P_2, and many studies have employed GFP fusions of PtdIns(3,4,5)P_3-specific or PtdIns(3,4,5)P_3/PtdIns(3,4)P_2-specific PH domains to monitor the accumulation and localization of PI 3-kinase products upon cell stimulation. It is generally assumed that phosphoinositides alone define the location of the GFP/PH domain fusions used in these studies, although other influences have not been excluded and there are several reasons to suspect that other (poorly defined) binding targets may also play a role [54].

17.3.1.1 PtdIns(4,5)P_2-specific PH Domains

PH domains from phospholipase C-δ isoforms (and related proteins) [31, 32, 56, 57] are the only known mammalian examples that are specific for PtdIns(4,5)P_2. In *S. cerevisiae*, as mentioned above, only the Num1p PH domain is PtdIns(4,5)P_2-specific [36]. The PH domain of PLC-δ_1 is located at its amino terminus and can bind both the substrate (PtdIns(4,5)P_2) and the product (Ins(1,4,5)P_3) of this enzyme. It is thought that binding of the PH domain to PtdIns(4,5)P_2 serves to anchor the whole enzyme to PtdIns(4,5)P_2-rich membranes, thus allowing processive or scooting-mode hydrolysis of substrate by PLC-δ_1 in these membranes [58, 59]. Binding of the soluble product (Ins(1,4,5)P_3) to the PH domain competes with PtdIns(4,5)P_2 binding [32], thus dissociating PLC-δ_1 from the membrane and inhibiting its activity [60, 61]. Through this mechanism, the PH domain influences PLC-δ_1 activity in a manner that depends on the ratio of PtdIns(4,5)P_2 and Ins(1,4,5)P_3 concentrations that it experiences.

The PtdIns(4,5)P_2-specific *S. cerevisiae* Num1p PH domain is required to anchor this protein to the mother cell cortex, so that it can serve as a cortical anchor for dynein as it drives nuclear migration through the bud neck during mitosis [62]. There is no reason to expect that Ins(1,4,5)P_3 binding is important for the Num1p PH domain.

Another very intriguing role for a PtdIns(4,5)P_2-binding PH domain was recently described for the Unc104 kinesin motor [63]. Unc104 has a PH domain, although one for which phosphoinositide binding specificity has yet to be determined. Klopfenstein et al. [63] found that Unc104 binds PtdIns(4,5)P_2-containing vesicles through this PH domain and uses its motor domain to transport these vesicles along microtubules. This finding illustrates the fact that we should bear in mind that PH domains can dock membranes (or lipid molecules) to proteins just as well as phosphoinositide-containing membranes can dock or recruit PH domain-containing proteins.

17.3.1.2 PI 3-kinase Product-binding PH Domains

Whereas membrane targeting by the very few PtdIns(4,5)P_2-specific PH domains is constitutive, it is directly signal-dependent for PH domains that recognize PI 3-kinase products. Please see the excellent reviews of PI 3-kinase signaling [40, 64, 65]. Conceptually, the role played by PH domains in responding to PI 3-kinase activation is very simple and is illustrated in Figure 17.4 for signaling via Akt–protein kinase B (PKB) and phosphoinositide-dependent kinase-1 (PDK1) [66]. After PI 3-kinase activation, PKB is rapidly recruited to the plasma membrane [67, 68] by virtue of the specific interaction of its N-terminal PH domain and the PtdIns(3,4,5)P_3 (and/or PtdIns(3,4)P_2) that transiently accumulates. This signal-dependent membrane recruitment can be reconstituted with the isolated PH domain and can be visualized directly (and dramatically) when the PH domain is fused to GFP [69–71]. Membrane recruitment of PKB brings it close to PDK1, which itself has a PI 3-kinase product-specific PH domain at its C terminus [72, 73]. There is disagreement as to whether PDK1 is frankly recruited to the plasma membrane by PI 3-kinase products [74, 75]. However, binding of both PKB and PDK1 to the same phosphoinositides would certainly enhance their colocalization, and under these conditions PDK1-mediated phosphorylation of PKB, which is required for PKB activation, would be promoted. PKB is phosphorylated in its activation loop (at T308) by PDK1 and becomes fully activated when it is also phosphorylated at S473 by another uncharacterized kinase [66] (or possibly by autophosphorylation [76]). The role of the PH domains in this pathway is to colocalize two proteins transiently, and in a regulated manner, so that one can phosphorylate the other and cause its signal-dependent activation. There are significant parallels between

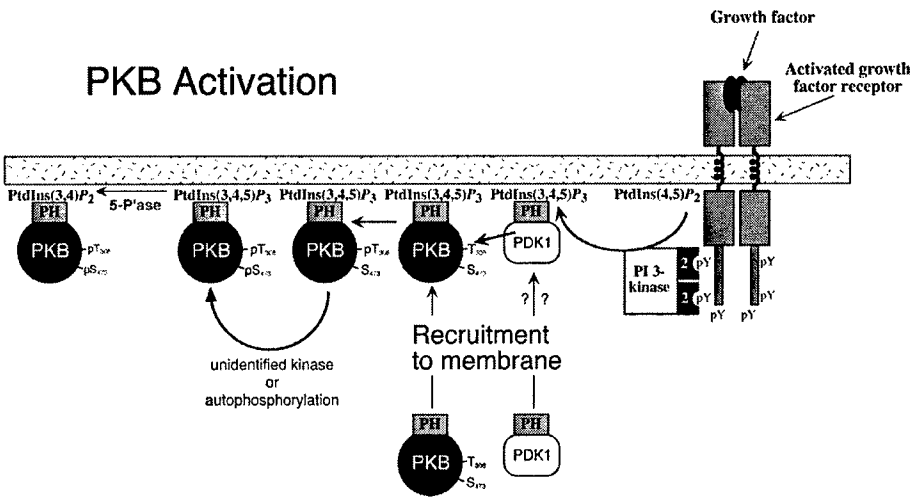

Figure 17.4 Role played by PH domains of PKB and PDK1 in PI 3-kinase signaling. A scheme for activation of PKB by growth factor-induced PI 3-kinase activation is shown. See text for details.

the roles that PH domains play in this pathway and those played by SH2 and PTB domains in other related pathways, as discussed in other chapters. Other PH domains that bind strongly and specifically to PI 3-kinase products function similarly. For example, by recognizing PtdIns(3,4,5)P_3, the PH domain of Btk brings this tyrosine kinase close to the membrane-associated Lyn tyrosine kinase that initiates Btk activation [77]. Grp1 is a guanine nucleotide exchanger for ADP ribosylation factor-6 (ARF6) at the plasma membrane. PtdIns(3,4,5)P_3-dependent recruitment of Grp1 to ARF6 at the plasma membrane activates this small G-protein, thus promoting cytoskeletal and membrane changes [77]. Other examples are detailed in the reviews of PI 3-kinase signaling cited above.

Although simple PI 3-kinase-dependent membrane recruitment seems sufficient to explain these signaling events, it has been speculated that an important conformational change in PKB is induced upon ligand binding to the PH domain. PKB mutants lacking the PH domain are reportedly more readily phosphorylated by PDK1 than is the intact protein [78], leading to the suggestion that the unliganded PH domain may shield T308 from PDK1 phosphorylation. A comparison of the crystal structures of unliganded and Ins(1,3,4,5)P_4-bound PKBα PH domain has suggested that this PH domain is unique in undergoing significant ligand-dependent conformational changes in the β1/β2, β3/β4 and β6/β7 loops [79]. The authors of this study suggested that these changes could disrupt intramolecular interactions that inhibit T308 phosphorylation and that this could be an important element of PI 3-kinase regulation of PKB. While this certainly is possible, we should note that the crystallization conditions used for the 'unliganded' and 'liganded' structure determinations began at pH values of 8.5 and 4.6, respectively, which could explain some of the observed structural differences. Moreover, a recent NMR study of the PKBβ PH domain (at pH 7.4) showed that the structures of all three of these loops (especially the β3/β4 and β6/β7 loops) are very ill defined – as seen for other PH domains – and no strong alteration in the dynamic behavior of these loops upon Ins(1,3,4,5)P_4 binding was detected by NMR [80]. It therefore remains unclear whether binding of PI 3-kinase products to the PKB PH domain promotes allosteric changes of the sort suggested by Milburn et al. [79] or whether these changes simply reflect different static structures trapped in different crystals that were grown at different pH values. However, even without significant, well defined ligand-induced changes in the PH domain itself, it does not seem unreasonable to suggest that reorientation of the PH and kinase domains at the membrane surface could promote the ability of PDK1 to phosphorylate PKB. Structural studies of the entire PKB molecule are required to address this question satisfactorily.

17.3.1.3 Membrane Targeting by PH Domains with Little Phosphoinositide-binding Specificity

A third group of PH domains that are clearly membrane targeted when analyzed in isolation (i.e., when expressed in cells as GFP-fusion proteins) has recently been identified. These bind phosphoinositides more than 10 times more weakly than the PLC-δ_1 PH domain or the PI 3-kinase product-specific PH domains. In addition, they show little or no stereospecificity in their phosphoinositide binding, interacting

with all known phosphoinositides with approximately the same affinity. Levine and Munro were the first to identify a PH domain having these characteristics [52, 81] – that from the oxysterol binding protein (OSBP). The OSBP PH domain is specifically targeted to the golgi complex in both yeast and mammalian cells when analyzed as a GFP-fusion protein [81]. Several PH domains with closely related sequences, including those from FAPP1 [81, 82], and the *S. cerevisiae* OSBP homologs Osh1p [36, 83] and Osh2p [36] are all similarly localized to the golgi. Most interestingly, the specific localization of all of these PH domains is largely abolished if golgi production of PtdIns(4)P is impaired, in a *pik1*^{ts} yeast strain [36, 52, 81, 82]. By contrast, decreased concentrations of PtdIns(4,5)P_2 or other phosphoinositides, or (most intriguingly) even decreased PtdIns(4)P production at the plasma membrane (in an *stt4*^{ts} yeast strain [84]) have no influence on the golgi localization of these PH domains. All of these golgi-located PH domains bind equally well to all phosphoinositides in vitro [36, 52], yet in vivo they bind to PtdIns(4)P only at the golgi. The most reasonable explanation for these observations is that golgi localization requires two targets: PtdIns(4)P and a second unidentified binding partner. The second binding partner is likely to be present only in the golgi, thus defining the specific localization of these PH domains, and is thought to cooperate with golgi phosphoinositides (presumably mostly PtdIns(4)P) to recruit the PH domains from OSBP, FAPP1, and Osh proteins. Levine and Munro identified yeast Arf1p as one candidate for this second component that may cooperate with PtdIns(4)P in recruiting the OSBP PH domain to the golgi [52].

A recent genome-wide analysis of *S. cerevisiae* PH domains [36] identified at least four plasma membrane-targeted PH domains that may have similar characteristics. These PH domains (from Cla4p, Skm1p, Yil105cp, and Ynl047cp) are targeted to the plasma membrane in a manner that depends on PtdIns(4,5)P_2 production. However, the in vitro PtdIns(4,5)P_2 binding affinities of these PH domains are too weak for this to be solely responsible for the observed in vivo membrane targeting. We suggest that these PH domains are targeted to the plasma membrane by simultaneously binding PtdIns(4,5)P_2 and another, as yet unidentified, target. The probable existence of other such non-phosphoinositide targets is further suggested by one yeast PH domain identified in this study [36] (from Opy1p) that binds phosphoinositides very weakly in vitro, yet is efficiently targeted to the plasma membrane in a manner that does not depend on in vivo phosphoinositide production.

17.3.2
Function of Low-affinity PH Domains That Are Not Independently Membrane Targeted

As mentioned in several places in this chapter, the majority of PH domains neither bind tightly (or specifically) to phosphoinositides nor interact strongly with cellular membranes. Understanding the functions of these PH domains is currently the greatest challenge. Many have been reported to bind phosphoinositides with very low affinities (and usually no specificity). Indeed, in *S. cerevisiae*, all that is known about 17 of the 33 PH domains is that they bind phosphoinositides in lipid-overlay experiments (but not strongly enough to detect by other methods) and that they

are cytoplasmic when expressed in yeast or mammalian cells as GFP fusions [36]. A similar conclusion has also been reached for the majority of mammalian PH domains that have been studied [46, 85], although several have also been reported to have protein targets [13].

The first question to address in light of these findings is whether or not the low-affinity phosphoinositide binding that is seen in vitro for nearly every PH domain has in vivo relevance. Indeed, it is troubling to realize that most PH domains (and many other proteins) can be purified very effectively by using cation exchange resins and that the binding matrices used to analyze phosphoinositide binding bear significant resemblance to these negatively charged resins. In other words, it actually seems quite likely that the low affinity and nonspecific phosphoinositide binding reported for many PH domains is actually an in vitro artifact. Contrary to this rather pessimistic view, it does appear that low-affinity phosphoinositide binding is important in at least some of these proteins – in particular for the PH domains from dynamin, a large GTPase involved in receptor-mediated endocytosis [86–88], and for the large group of PH domains that immediately follow Dbl homology (DH) domains in guanine nucleotide exchange factors (GEFs) for Rho GTPases.

17.3.2.1 The Dynamin PH Domain

Dynamin is a large GTPase (with one PH domain) that assembles at the necks of invaginated coated pits during receptor-mediated endocytosis and is intimately involved in the scission of these necks to produce endocytic vesicles [89]. PtdIns(4,5)P_2 and other phosphoinositides enhance the in vitro GTPase activity of dynamin [37, 90] in a way that appears to require its oligomerization into well defined assemblies [91, 92]. Moreover, although the monomeric dynamin PH domain has a very low affinity for phosphoinositides, it binds quite strongly to PtdIns(4,5)P_2-containing membranes when oligomerized (as in self-assembled dynamin) [93]. Thus, dynamin self-assembly and PH domain-mediated membrane binding appear to be thermodynamically coupled.

Expression of full-length dynamin with a phosphoinositide-binding–defective PH domain has a dominant negative effect on the function of endogenous dynamin in HeLa and other cells [86–88]. Dynamin assemblies containing some molecules with defective PH domains are therefore thought to be incapable of interacting sufficiently strongly with PtdIns(4,5)P_2-containing membranes for dynamin's function to be executed. In other words, overexpressed PH domain-mutated dynamin appears to 'poison' the avidity advantage afforded to the PH domain by self-assembly. Details of the step (or steps) in which PtdIns(4,5)P_2 binding to the dynamin PH domain is functionally important for its role in the scission of endocytic vesicles remain very poorly defined. However, the evidence for a requirement that multiple PH domains within a dynamin oligomer cooperate with one another to fulfill this role appears to be quite strong.

17.3.2.2 PH Domains of Dbl-family Proteins

The PH domains that follow Rho GEF/Dbl homology domains (referred to here as DH domains) in Dbl family proteins [94] are another group for which low-affinity,

often promiscuous, phosphoinositide binding appears to be functionally important. The Dbl family proteins are guanine nucleotide exchange factors (GEFs) for Rho-family small GTPases, and the 200 amino-acid DH domain is necessary and sufficient for their GEF activity [94, 95]. In mammalian Dbl family proteins the DH domain is almost invariably followed by a PH domain, and the 46 or more examples for which this is true account for some 18% of all human PH domains. In all instances studied, DH-associated PH domains bind phosphoinositides with low affinity (K_D values > 10 μM), and in most examples, binding appears to be promiscuous [13, 96]. A series of recent publications have shown that mutations that impair the low-affinity in vitro phosphoinositide-binding interactions of these PH domains also impair the in vivo GEF activity of the full-length proteins [97–104]. Consistent with their low-affinity binding to phosphoinositides, neither isolated Dbl family PH domains nor DH/PH fragments of these proteins appear to be independently targeted to cellular membranes. By contrast, full-length Dbl family proteins are often found to be membrane-associated, most likely as a consequence of interactions mediated by the multiple other domains that they contain. It seems likely that phosphoinositide binding by the PH domain cooperates with these other domains in altering the extent or specific location of membrane targeting. PH domain mutations that impair the function of intact Dbl family proteins have been reported to alter subcellular localization in some mutants [101, 103] but not significantly in others [97, 98, 100].

PH domains that follow DH domains may also play a role that does not involve driving membrane targeting, which may be their primary role. In the context of a membrane-associated multidomain protein, the PH domain could direct localization to membrane regions that are rich in phosphoinositides, which could be important for colocalizing the Dbl family proteins with its small GTPase targets. The specificity of the C-terminal Tiam-1 PH domain for PtdIns(3)P [96, 98] suggests such a 'lateral targeting' possibility, although this remains speculative. Structural studies of DH/PH fragments with and without bound Rho-family GTPases suggest an additional possibility [105–108]. In the structure of the Sos DH/PH fragment, the PH domain is positioned so that it prevents access of the small GTPase to its binding site on the DH domain [105]. In the structures of DH/PH fragments bound to small GTPases [106–108], the PH domain is oriented quite differently, so that the small GTPase has access to the DH domain. It has been proposed that phosphoinositide binding to the PH domain (at a membrane surface) promotes reorientation of the two domains so that the PH domain no longer interferes with access to the DH domain binding site, thus promoting exchange activity of the Dbl family protein [97, 105, 107]. The structure of the Dbs DH/PH fragment bound to cognate GTPases revealed additional unexpected direct contacts between the PH domain and the bound Cdc42 [106] or RhoA [108]. It is possible that phosphoinositide binding to the PH domain promotes an orientation of the DH and PH domains at the membrane surface that is ideal for these critical interactions. Thus, in the Dbl family proteins, PH domains may play roles in both membrane recruitment (either bulk localization or lateral targeting to specific regions of the membrane) and allosteric regulation by altering the relative orientations of adjacent domains.

17.3.3
Protein Targets of PH Domains

It is difficult to write about protein targets of PH domains – of which many have been suggested – without the risk of simply producing a list. However, two themes appear to be emerging. One was touched upon in the Introduction and involves PH domain interaction with the βγ subunits of heterotrimeric G proteins. The other was introduced in the previous section by the fact that the PH domain of Dbs directly contacts Cdc42 or RhoA bound to the DH/PH fragment of this Rho family GEF.

17.3.3.1 Small GTPases as PH Domain Targets

There have now been several reports of PH-domain interactions with small GTPases, and the surprising finding that the first Ran-binding domain (RanBD1) from Ran-binding protein-2 (RanBP2) closely resembles a PH domain [24] provides a conceptual framework for this. RanBP2 binds specifically to the GTP-bound form of the small GTPase Ran (involved in regulation of nucleocytoplasmic transport), using a binding site that contains its three PH-domain variable loops [24].

In addition the above reports, three other reports provide compelling evidence for GTP-regulated interaction between PH domains and small GTPase targets. Jaffe et al. [109] recently showed that the PH domain of hCNK, the human homolog of connector enhancer of ksr (kinase suppressor of ras), interacts with Rho. Although the interaction appears to be relatively weak (and may require cooperation with other domains in CNK), it appears to be selective for the GTP-bound form of Rho (as in RanBP2–Ran interactions). Similarly, Kim et al. [110] presented evidence to suggest that Etk, a tyrosine kinase from the Btk family, interacts specifically with GTP-bound RhoA through the N-terminal Etk PH domain. A third example of this, suggested from genetic studies, was outlined above. In analyzing golgi localization of the OSBP PH domain, Levine and Munro [52] provided evidence that the small GTPase Arf1p may cooperate with phosphoinositides in recruiting PH domains to this organelle.

A more thorough and quantitative analysis has been applied to the interaction between the N-terminal PH domain of PLC-β_2 and Rac GTPases [111]. Sondek and colleagues analyzed PLC-β_2 binding by 17 different GTP-bound members of the Rho family and found that only the GTP-bound form of the three Rac GTPases bound significantly to (and activated) PLC-β_2. Binding of GTP-bound Rac GTPases to the isolated PLC-β_2 PH domain had essentially the same affinity (within a factor of two) as their binding to full-length PLC-β_2 in surface plasmon resonance studies, with K_D values in the 5–10 μM range. These more detailed studies add substantial credibility to the increasing number of other reports that certain PH domains bind to small GTPases and, by binding only to their GTP-bound state, may serve as Rac, Rho, or Arf1p effectors. This class of protein interaction is the closest that PH domains have come to having a common protein target.

17.3.3.2 **Other Protein Targets of PH Domain Targets**

We previously reviewed many of the proposed protein targets for PH domains that are based on one or two reports [13] and for which the physiological relevance is not completely clear. Rather than repeat this, here we summarize suggested targets by group and provide one or two examples. One of the difficulties in considering protein targets of PH domains is that the studies proposing them often rely solely on qualitative coprecipitation experiments and often use deletion mapping to implicate the PH domain – without accounting for the possible effects of deletions on overall protein conformation and stability.

The first class of proposed PH domain ligands are $G_{\beta\gamma}$ subunits of heterotrimeric G proteins [7], which initially attracted attention when it was appreciated that the site on βARK that interacts with $G_{\beta\gamma}$ is part of the βARK PH domain [112, 113]. The interaction of PH-domain–containing G protein-coupled receptor kinases (GRKs) (including βARK) with $G_{\beta\gamma}$ subunits has a well studied role in recruiting the kinase to the cell membrane after GPCR activation, so that it may phosphorylate and thus initiate down-regulation of the receptor [114]. Furthermore, a recent crystal structure has shown precisely how the GRK2/βARK PH domain associates with the $G_{\beta\gamma}$ subunit (Figure 17.5b), as discussed in Section 17.4. From this structure [11], it is clear that direct interaction of the PH domain with the membrane-associated $G_{\beta\gamma}$ subunit can cooperate with simultaneous PtdIns(4,5)P_2 binding by this PH domain [10] to drive its membrane targeting, possible also allosterically activating the GRK2 kinase domain. Apart from this clear and well-characterized example, however, no convincing physiological role for PH domain–$G_{\beta\gamma}$ interactions has been established, and no others have been characterized quantitatively.

The second class of potential PH domain protein targets that were suggested early on are protein kinase C (PKC) isoforms – appropriate, since pleckstrin is the major PKC substrate in platelets [115]. These examples are listed elsewhere [13], and their physiological roles remain to be established. In addition, a G_{α} subunit [116], filamentous actin [117], myosin II [118], the TCL1 oncogene product [119], and a transcription factor TFII-I [120] have all been reported to bind to one or more PH domains. However, no common theme emerges with regard to the structural basis or functional importance of these interactions, and much more investigation is needed. Finally, an intriguing set of results was reported from yeast two-hybrid studies of the IRS-1 PH domain, which demonstrated that this domain recognizes regions of protein sequence rich in acidic residues [121] (nucleolin being one example). In our own early studies of the Ras-GAP PH domain, a very similar sequence (in Nopp140) was identified as a preferred protein ligand in screens of bacterial expression libraries (M. A. L. and J. Schlessinger, unpublished). Although the relevance of these interactions is not clear, these studies do raise the possibility that there may be particular protein sequence motifs recognized by subsets of PH domains, which warrants further investigation.

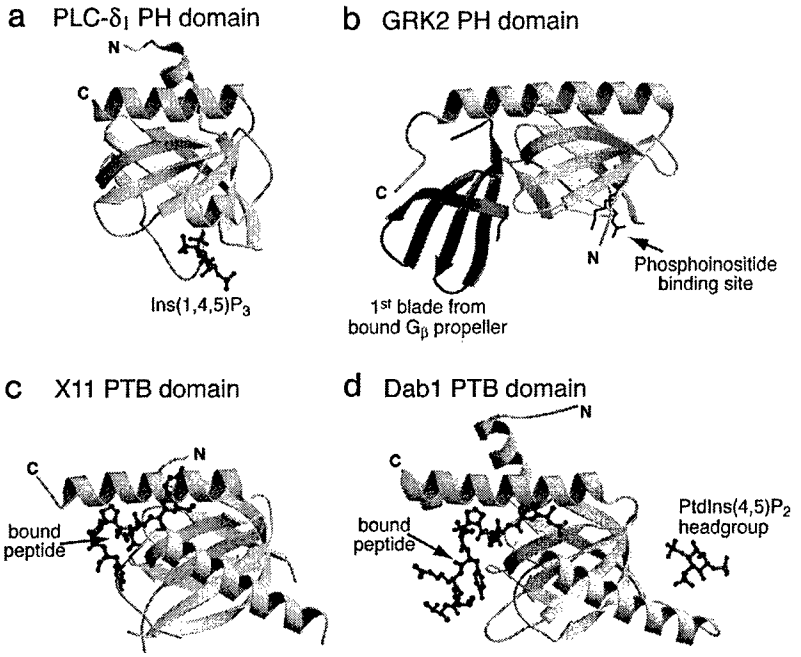

Figure 7.5 Examples of multiple ligands binding simultaneously to a single PH-related domain. (a) The PH domain from PLC-δ_1 [15] is shown bound to Ins(1,4,5)P_3 as an example of a singly liganded PH domain. (b) The PH domain from GRK2 is shown, with the first blade from the G_β propeller that interacts with the C-terminal part of the PH domain helix and strands β2 through β4 [11]. The phosphoinositide binding site on the GRK2 PH domain, identified by NMR [39], is also noted. This PH domain represents an example in which protein and phosphoinositide ligands can interact with the same PH domain simultaneously to drive membrane recruitment (see text). (c) The X11 PTB domain is shown [125], bound to a peptide modeled on a region of the amyloid precursor protein. The peptide lies in a groove between strand β5 and the C-terminal α helix, as seen for all PTB domain–peptide interactions [122]. This represents a 'classical' PTB domain interaction. (d) The structure of the disabled-1 PTB domain is shown [123], with a bound NPXY-containing peptide that interacts in a manner that is very similar to peptide binding to X11-PTB. In addition, the Dab1 PTB domain has a bound PtdIns(4,5)P_2 headgroup molecule, located relatively close to the phosphoinositide-binding site of a 'canonical' PH domain. This complex represents an example of how proteins and phosphoinositides can cooperate in driving membrane association of a PTB domain, which combines PTB-like and PH-like characteristics (see text).

17.4
Emerging Research Directions and Recent Developments

In our efforts to understand the function of PH domains, it is possible that things have been made more difficult by the (perhaps somewhat arbitrary) distinction between different subgroups of domains that contain the PH domain fold. It is true that sequence comparisons do not readily identify a PTB domain, for example, with PH domain sequence profiles. However, it is also true that there can be more sequence identity between a PTB domain and a PH domain than between two different PH domains. It is therefore worthwhile to consider domains with the PH domain fold as a whole when considering possible functions. Among the domains with the PH domain fold, there are subgroups that bind proline-rich sequences (EVH1 domains [25]), phosphorylated or β-turn peptides (PTB domains [122]), small GTPases (RanBD1 [24]), $G_{\beta\gamma}$ subunits (GRK2 [11]), and specific phosphoinositides (PH domains with the β1/β2 loop motif shown in Figure 17.3). There may well be other categories that have yet to be identified. However, there may be many examples that combine features from more than one of these categories. Indeed, several PH domains were discussed above, some identified in yeast, that appear to interact weakly with phosphoinositides and weakly with a second target – cooperation between the two targets being required for efficient membrane recruitment. In these instances it may be difficult to convincingly identify either target on its own because of low binding affinities, and new approaches for context-dependent screens may be needed. Figure 17.5 gives a structural impression – based on examples that are already well understood – of how such multiligand PH domains may function. On the left side is shown the PH domain of PLC-δ_1, with Ins(1,4,5)P_3 in its binding site (Figure 17.5a), and the X11 PTB domain with an NPXY peptide in its binding site that lies between strand β5 and the C-terminal α helix (Figure 17.5c). Recent studies of the PTB domain from disabled-1 (Dab1) and disabled-2 (Dab2) have shown how this domain with a PH fold can simultaneously exhibit features of a PTB domain and a PH domain [123, 124]. The Dab1 PTB domain binds its cognate peptide just as other PTB domains are seen to do (Figure 17.5d). In addition, it binds the headgroup of PtdIns(4,5)P_2 in a positively charged region that is similar in nature to the PtdIns(4,5)P_2 binding site of the PLC-δ_1 PH domain and is located broadly on the same face. The Dab PTB domains are thought to bind simultaneously to intracellular protein sequences in LDL receptor family members and to phosphoinositides to recruit them to the plasma membrane [123]. A second, more PH-like, solution to this problem is seen with the GRK2 PH domain that was discussed in Section 17.3.3.2. The structure of this PH domain, taken from the GRK2–$G_{\beta\gamma}$ complex structure [11] is shown in Figure 17.5b. The phosphoinositide-binding site, identified in NMR studies by Cowburn and colleagues [39], is marked. This corresponds well with the Ins(1,4,5)P_3 binding site in the PLC-δ_1 PH domain. Figure 17.5b also shows the first blade of the G_β propeller, which contains all the sites that contact the GRK2 PH domain. The binding site for G_β involves the C-terminal part of the PH domain helix (extended in GRK2-PH) and lies on the opposite side of this helix from the peptide binding site seen in PTB domains.

Whereas the peptide bound to PTB domains lies along the length of strand β5, G_β contacts the beginning of strand β1 and the C termini of strands β2 and β4. The locations of the binding sites for G_β and phosphoinositides on the GRK2 PH domain explain how these two ligands can cooperate to drive membrane targeting of this protein [10]. These examples represent one instance in which it could be argued that a phosphoinositide-binding PH domain has acquired the capacity to interact with additional protein targets (GRK2-PH), and another in which a PH-related protein binding domain has acquired phosphoinositide binding capacity (Dab1-PTB). We suggest that many other examples of this sort exist, in which phosphoinositide and protein (or two different proteins) are recognized simultaneously by the same domain. Identifying these domains and their binding targets will provide the next chapter in our quest to understand PH domain function.

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